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~~The role of polyamines in *Arabidopsis*: effects on stress  
tolerance and on plant development~~

THE FUNCTION OF POLYAMINES IN  
ARABIDOPSIS THALIANA

A thesis submitted for the degree of Doctor of Philosophy

by

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## Abstract

To evaluate the importance of polyamines under stress conditions, mutants in the polyamine pathway were grown under salt and osmotic stress. Two types of mutant were chosen, methionine-overproducing (*mto*) mutants (may increase polyamine production) and arginine decarboxylase (*spe*) mutants (may decrease polyamine production). Initial experiments were done to determine how polyamines were to be extracted from the plant. Under normal growth conditions, the polyamine content of each mutant was similar to wild type contents. Polyamines varied in different organs of *Arabidopsis* with flowers having higher polyamine contents than green organs. To determine the effect of stress on the mutants,  $F_v/F_m$  (maximum photosynthetic efficiency), fresh weight, chlorophyll and polyamine contents were determined. Under salt stress, polyamine contents increased in wild type plants, but only when they were germinated on salt-containing medium. *spe* mutants showed a decreased tolerance to saline conditions, and the *spe2-1* mutant plants also showed a decrease in polyamine accumulation compared to its wild type. Under severe salt stress, *spe* mutants bleached more rapidly than their wild types. Results for the effect of salt stress on the *mto* mutants were inconsistent. Under osmotic stress (treatment with polyethylene glycol), polyamine content decreased and no clear difference was found between the effect on *mto* mutants and their wild types. To isolate mutants in polyamine signalling, activation-tagged lines were screened on Murashige-Skoog (MS) medium supplemented with 5 mM spermidine. Mutants that stayed green under these conditions and/or had longer roots than the wild type were isolated. The mutants were crossed to the wild type and the  $F_2$  generation was analysed for segregation on BASTA and on spermidine-containing medium. Adaptor PCR, inverse PCR and plasmid rescue were used to determine the insertion sites of the enhancer tag. One of the mutants had several T-

DNA inserts, one next to a 5S rRNA gene and one in either a gene for a chloroplast nucleoid DNA-binding protein or a U2 small nuclear ribonucleoprotein. It is therefore unclear what caused the mutant phenotype.

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*For Nigel*

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## Abbreviations

Abbreviation	Full Name
ABA	Absciscic acid
ADC	Arginine decarboxylase
CGS	Cystathionine- $\gamma$ -synthase
DAO	Diamine oxidase
DFMO	$\alpha$ -difluoromethylornithine
EMS	Ethyl methanesulfonate
HCAA	Hydrocinnamic acid amide
MS	Murashige-Skoog
ODC	Ornithine decarboxylase
PAO	Polyamine oxidase
PA	Polyamine
PEG	Poly-ethylene glycol
Put	Putrescine
SAM	S-adenosyl methionine
SAMDC	SAM decarboxylase
Spm	Spermine
SPMS	Spermine synthase
Spd	Spermidine
SPDS	Spermidine synthase
TS	Threonine synthase



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## **Chapter One: Introduction**

## **1.1 Polyamines**

Polyamines are composed of only carbon, nitrogen and hydrogen and contain two or more amino groups. The most common plant polyamines are the diamine putrescine (1,4-diaminobutane), triamine spermidine (1,8-diamino-4-azaoctane) and tetramine spermine (1,2-diamino-4,9-diazadodecane). Other polyamines include the diamines 1,3-diaminopropane, norspermine, norspermidine, caldopentamine, homocaldohexamine and cadverine (1,5-diaminopentane). Polyamines are polycationic compounds. They do not only exist in plants but are present in all living organisms. Polyamines can be conjugated to phenolic acids and, due to their positive charge, have been found bound to macromolecules, such as proteins. Although some researchers believe that polyamines are hormones, others have suggested that they act as hormonal second-messengers (Slocum and Flores 1991).

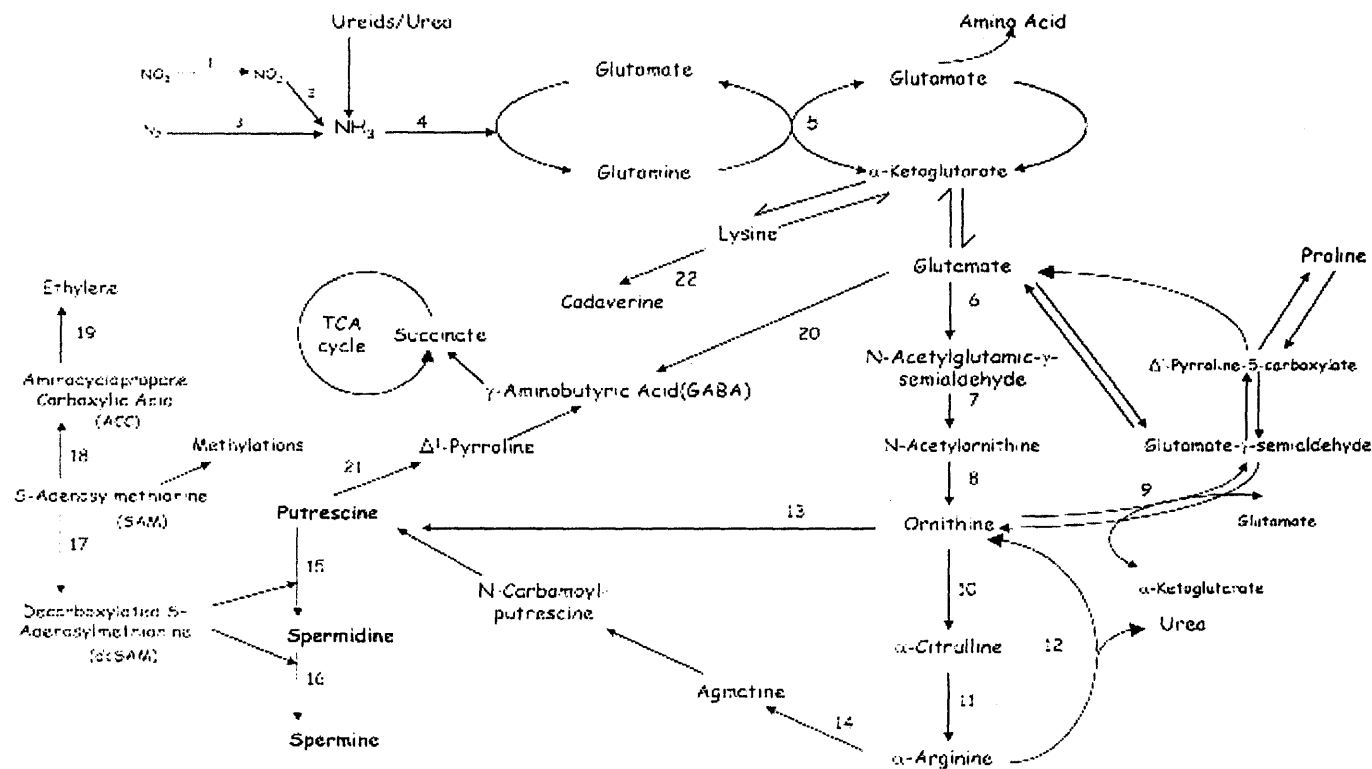
## **1.2. Polyamine biosynthesis**

The diamine putrescine can be formed from ornithine by the enzyme ornithine decarboxylase (ODC, EC 4.1.1.17) or by the decarboxylation of arginine by arginine decarboxylase (ADC, EC 4.1.1.19). As shown by





, spermidine and spermine are made from putrescine by the addition of aminopropyl groups, catalyzed by the aminopropyl transferases spermidine synthase (SPDS, EC 2.5.1.16) and spermine synthase (SPMS, EC 2.5.1.22). These aminopropyl groups are obtained from decarboxylated *S*-adenosylmethionine, created by the enzyme *S*-adenosylmethionine decarboxylase (SAMDC, EC 4.1.1.50). Polyamines have been localised in the vacuole, mitochondria and chloroplasts (Slocum *et al.* 1991) Generally ODC, SAMDC and spermidine synthase localise to the cytoplasm (Tiburcio *et al.* 1990). The polyamine content of plants is regulated at the transcriptional, post-transcriptional, translational and post-translational levels (Cohen 1998). The activity of polyamine biosynthetic enzymes can be regulated by polyamines or other hormones. For instance, ADC activity is inhibited by spermine (Borrell *et al.* 1996) and its nucleotide sequence has ethylene response elements within it. SAMDC activity decreases in plants treated with exogenous spermidine (Tassoni *et al.* 2000). Although both ADC and SAMDC are regulated at the post-translational level (Kumar *et al.* 1997), this may not be the case with all polyamine biosynthetic enzymes. For instance, SPDS has been shown to have a different regulation to SAMDC (Tassoni *et al.* 2000). Although the regulation of polyamine synthesis and catabolism is still being investigated, it is clear that polyamines are tightly regulated, as many transgenic plants with a high increase in enzyme activity show no correlating accumulation of polyamines (Mayer and Micheal 2003).



**Figure 1.** Polyamine biosynthesis. Polyamine biosynthesis and related nitrogen metabolism. The enzymes are: 1, nitrate reductase; 2, nitrite reductase; 3, nitrogenase; 4, Gln synthetase (GS); 5, Glu synthase (GOGAT); 6, Glu reductase; 7, acetylglutamic- $\gamma$ -semialdehyde transaminase; 8, acetylornithinase; 9, Orn aminotransferase (OAT); 10, Orn transcarbamylase; 11, Arg synthase; 12, arginase; 13, Orn decarboxylase (ODC); 14, Arg decarboxylase (ADC); 15, spermidine synthase; 16, spermine synthase; 17, SAM decarboxylase (SAMDC); 18, ACC synthase; 19, ACC oxidase; 20, Glu decarboxylase (GAD); 21, diamine oxidase; and 22, Lys decarboxylase (LDC). Polyamine biosynthesis and related nitrogen metabolism. The enzymes are: 1, nitrate reductase; 2, nitrite reductase; 3, nitrogenase; 4, Gln synthetase (GS); 5, Glu synthase (GOGAT); 6, Glu reductase; 7, acetylglutamic- $\gamma$ -semialdehyde transaminase; 8, acetylornithinase; 9, Orn aminotransferase (OAT); 10, Orn transcarbamylase; 11, Arg synthase; 12, arginase; 13, Orn decarboxylase (ODC); 14, Arg decarboxylase (ADC); 15, spermidine synthase; 16, spermine synthase; 17, SAM decarboxylase (SAMDC); 18, ACC synthase; 19, ACC oxidase; 20, Glu decarboxylase (GAD); 21, diamine oxidase; and 22, Lys decarboxylase (LDC). From Bhatnagar *et al.* (2001)

The importance of the ODC and ADC pathways is reflected through the change in plant morphology after the use of enzyme inhibitors. ADC inhibitors cause different effects to ODC inhibitors, which indicate that, although these enzymes lead to the same products, they have different roles. It has been postulated that the ODC pathway is more important in cell division, whereas the ADC pathway is involved in environmental responses. ADC and ODC activities have been measured in most plants, but the existence of the ODC pathway has been debated in *Arabidopsis* (Urano *et al.* 2003). Although some research groups have detected ODC activity in *Arabidopsis* (Feirer *et al.* 1998, Tassoni 2000), other groups have stated that *Arabidopsis* lacks the ODC enzyme and that ODC activity was due to a non-enzymatic decarboxylation of ornithine in the *Arabidopsis* extract (Hanfrey *et al.* 2001). In *Arabidopsis* there are two genes for ADC, four genes for SAMDC, two genes for SPDS and two genes for SPMS (Urano *et al.* 2003).

### 1.3 Arginine decarboxylase

The ADC genes are needed for plant reproduction and mutants that are deficient in both *ADC1* and *ADC2* will not produce viable seeds (Urano *et al.* 2005). In fact, seeds with the *ADC1*<sup>-/-</sup>*ADC2*<sup>-/-</sup> genotype could not be produced, reflecting the importance of the polyamine pathway in seed development. In the same paper it was stated that seedlings with an *ADC1*<sup>+/-</sup>*ADC2*<sup>-/-</sup> or *ADC1*<sup>-/-</sup>*ADC2*<sup>+/-</sup> genotype often had abnormal seed development. This shows that both these genes have important and unique roles in plant development. The *Arabidopsis* ADC genes, *ADC1* and *ADC2* are 80% identical (Urano *et al.* 2003), mainly differing in the amino-terminus. They are located on chromosomes II and IV respectively. Hanfrey *et al.* (2001) showed that ADC1 is a head to tail homodimer with two active sites, which may be created after a proteolytic

reaction between monomers. Interestingly, the TargetP programme (<http://www.cbs.dtu.dk/services/TargetP/>) localised ADC1 to the chloroplast. TargetP predicted ADC2 was not targeted in the chloroplast, mitochondria or secretory pathway. On the other hand, PSort (<http://psort.nibb.ac.jp/>) showed that ADC1 associates with the cytoplasm, microbodies and the mitochondrial matrix and that there is a high possibility that ADC2 is localised to the chloroplast stroma. However, ADC2 also showed an association with the chloroplast thylakoid membrane, the cytoplasm and microbodies (Emanuelsson *et al.* 2000). Although programmes are unclear on ADC localisation, Borrell *et al.* (1995) used immunoblotting to show that ADC is present in chloroplasts and is associated with the thylakoid membrane. Furthermore, Urano *et al.* (2003) showed that while ADC2 is expressed in flowers, buds, immature siliques and rosettes, ADC1 is expressed equally in all organs, but not at all in siliques.

For *ADC2*, mRNA has been shown to accumulate in ABA, methyl jasmonate and NaCl treatments, whereas *ADC1* mRNA does not (Perez-Amandor *et al.* 2002, Soyka *et al.* 1999). In fact, the *ADC1* and *ADC2* promoters have very different regulatory elements, which may lead to the specialisation of roles between the two enzymes. The *ADC2* promoter is strongly associated with seed germination, root and leaf development and is stimulated by light, sucrose and ethylene, whereas the *ADC1* promoter activity is low during vegetative development and is up-regulated after chilling (Hummel *et al.* 2004).

#### **1.4 S-Adenosyl-L-methionine decarboxylase (SAMDC)**

S-Adenosyl-L-methionine decarboxylase (EC 4.1.1.50) is an enzyme in the biosynthetic pathway of polyamines. Franceschetti *et al.* (2001) showed that *SAMDC1* is ubiquitously expressed and *SAMDC2* is expressed preferentially in leaves and inflorescences. This suggests different roles for these two genes, which opens the possibility that one responds to environmental factors. There are four additional



*SAMDC* genes within the *Arabidopsis* genome in addition to the two expressed *SAMDC* genes. No corresponding EST sequences for any of these four genes were found showing that they are unlikely to be expressed. All expressed plant *SAMDC* mRNA 5' leader sequences were shown to contain a highly conserved pair of overlapping upstream open reading frames (uORFs) that overlap by one base (Tian *et al.* 2004).

Polyamines can repress the translation of *SAMDC*. In the animal system, Hill *et al.* (1993) showed this to be mediated by the peptide product of the MAGDIS sequence (a small uORF in the 5' leader sequence of the mRNA). Polyamines bind to MAGDIS and cause ribosomes to stop translation (Hill *et al.* 1993). Franceschetti *et al.* (2001) stated that in plants the small uORF in the same position as MADGIS is too small to be likely to be recognized when *SAMDC* is translated. Therefore, they concluded that another mechanism of response to polyamines must occur. Hanfrey *et al.* (2002) contested this and showed that by deleting the upstream uORF of *SAMDC*, polyamine synthesis could be disrupted, causing abnormal plant development.

### **1.5 Spermidine synthase (SPDS) and Spermine synthase (SPMS)**

SPDS activity is essential for the growth of *Arabidopsis* (Imai *et al.* 2004), although it is not a rate-limiting step in spermidine synthesis (Franceschetti *et al.* 2004). There are two SPDS genes in plants, *SPDS1* and *SPDS2*. *SPDS* is expressed in most tissues, but is abundant in young tissue and in the vascular region. Kasukabe *et al.* (2004) showed that the up-regulation of *SPDS* in transgenic *Arabidopsis* leads to tolerance to different stresses and increased the expression of stress-induced genes. Spermidine production correlates with the tissue-specific regulation of *SAMDC* and *SPDS* in plants (Malmberg *et al.* 1998).

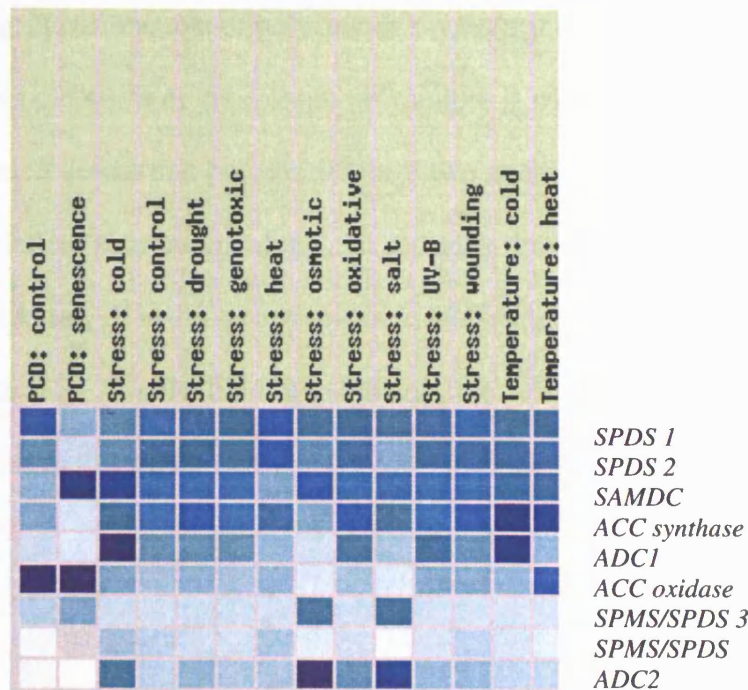
Although SPMS is expressed ubiquitously throughout the plant, its expression is not essential for survival (Imai *et al.* 2004). Without the SPMS genes, *Arabidopsis* suffers a severe defect in stem elongation. There are two genes encoding spermine synthase: *ACAULIS5* (*ACL5*) (Hanzawa *et al.* 1997, Imai *et al.* 2004) and *SPMS*. Panicot *et al.* (2002) showed that SPMS could interact with SPDS1 and SPDS2 forming SPDS1–SPMS and SPDS2–SPMS heterodimers *in vivo*. No interaction with ACL5 was found, so perhaps, by forming dimers with SPDS, SPMS has preference to receive spermidine. Furthermore, *SPMS* is upregulated by abscisic acid, which incidentally has no effect on *SPDS* (Hanzawa *et al.* 2000). This suggests that spermine production may be triggered under specific environmental factors.

### 1.6 Polyamine degradation

Polyamines are degraded by diamine oxidase and polyamine oxidase (which are located mainly in the apoplast), resulting in the formation of hydrogen peroxide ( $H_2O_2$ ). Putrescine is deaminated to make  $\Delta^1$ -pyrroline by the copper containing diamine oxidase (DAO, EC 1.4.3.6) releasing ammonia and hydrogen peroxide. Pyrroline dehydrogenase converts  $\Delta^1$ -pyrroline to GABA, which is transaminated and oxidised to create succinic acid, which enters the Krebs cycle. Spermine and spermidine are degraded by a flavoprotein-containing polyamine oxidase (PAO, EC 1.5.3.3). Degradation of spermidine yields  $\Delta^1$ -pyrroline and 1,3-diaminopropane and spermine gives 1,3-aminopropylpyrroline along with diaminopropane and  $H_2O_2$ .  $H_2O_2$  has been proposed to act as a signal molecule or as an antimicrobial compound in host resistance (Walters 2003).  $H_2O_2$  is converted into water and oxygen by plant catalases, located in peroxisomes and glyoxysomes (Xiong *et al.* 2002). This is important as  $H_2O_2$  is toxic and over-accumulation can lead to cell death (Yoda *et al.* 2003). Furthermore,



increase in polyamine content corresponded to a decrease in ethylene. They showed that polyamines can inhibit ethylene production, but ethylene can only inhibit polyamine production in deeply stressed leaves. Furthermore, transgenic potato plants with a low spermidine concentration have increased ethylene content (Kumar *et al.* 1996), suggesting a competitive relationship between ethylene and polyamines. However, this does not mean that their roles are antagonistic. For instance, Locke *et al.* (2000) showed that blocking ethylene production in barley enhanced free putrescine and spermidine content in grain, suggesting that, in the case of early growth, polyamines and ethylene hold complementary roles. In fact, there are ethylene response elements (ERE) in *ADC1* and *ADC2* (Hummel *et al.* 2004). In lettuce, Zapata *et al.* (2003) observed an increase in ethylene under salt stress, however no relation was found between this and salt tolerance. They showed that the activity of both ethylene and polyamine pathways increased under salt stress and that there was no competition between the metabolic pathways of ethylene and polyamines for SAM. The expression patterns of genes for ethylene and polyamine synthesis under different stress conditions are shown in Figure 2. It is important to compare the expression levels with the control values. Note that the PCD control corresponds to the senescence data and the stress control (in row 4) corresponds to the stress data. Both, genes for polyamine and ethylene synthesis are induced by stress. However, differences can be seen in the expression of *ADC2* (mainly induced by osmotic and salt stress) and of the ACC synthase gene (mainly induced by oxidative and temperature stress). This may suggest different roles of polyamines and ethylene during stress.



**Figure 2.** Levels of enzyme transcripts in plants grown under control conditions and a variety of stress conditions. The figure was produced by analysing Affymetrix GeneChip data from the AtGenExpress experiments using Genevestigator (<https://www.genevestigator.ethz.ch/>). The darker the blue colour, the stronger the expression of the gene.

If polyamine contents are affected under any conditions in the mutants studied in this thesis, this will have effects on many hormone responses, not only ethylene formation. For example, ABA has been linked to both the polyamine and ethylene pathway (Martínez-Madrid *et al.* 2004).

### 1.9 Function of polyamines

As early as 1984, it was shown that an increase in polyamines was linked to an increase in protein, DNA and RNA synthesis (Tabor 1984). Many studies later, the influence of polyamines on DNA/RNA and protein conformation and stability has been ascribed to more specific roles, such as the activation of the ribosomal ternary complex during protein synthesis and the polymerization of cytoskeletal components (Schuber 1989, Igarashi and Kashiwagi 2000). By affecting transcription and translation, polyamines have been linked to a variety of plant responses and are therefore considered important

in signalling. Thus, the role of polyamines has been shown to be diverse and difficult to pinpoint. An overview of the roles of polyamines is detailed in Table 1. By examining the literature, it seems that polyamines have two main, broad effects on plants. Firstly, polyamines affect plant morphology and secondly they affect plant responses to abiotic and biotic stresses. It is the second response that is the focus of this thesis. However, as stress response is a wide field, experiments are restricted to the effects of salt and osmotic stress in this thesis.

**Table 1. An overview of the many roles of polyamines in plants.**

<b>Role</b>	<b>Effect</b>	<b>Reference</b>
Parthenocarpy	PAs induce parthenocarpy	Fos <i>et al.</i> (2003)
Nutritional and vine life	Increased PAs increase nutritional quality, juice quality, and vine life of tomato fruit. Used enhanced SAMDC to increase both ethylene and polyamine accumulation.	Mehtha <i>et al.</i> (2002)
Root Length	Spermidine and spermine contents showed positive correlations with primary root growth, whereas the putrescine content showed neutral or negative effects on this trait	Hummel <i>et al.</i> (2002)
DNA transcription	PAs involved in the transcription of various growth-related genes.	Wang <i>et al.</i> (2002)
Growth	PAs may play a role in the regulation of postfertilization growth and development of reproductive organs.	Slocum <i>et al.</i> (1985)
Stomatal movement	PAs inhibit stomatal opening.	Liu <i>et al.</i> (2000)
Seed germination	PAs are involved in germination.	Sepulveda <i>et al.</i> (1988)
Cell division	PAs play a role in plant development.	Evans and Malmberg (1989)
RNA and DNA binding	PAs interact with nucleic acids.	Igarashi <i>et al.</i> (2000)
Flowering	PAs change during flowering.	Martin-Tanguy (1997)
Senescence	Exogenous PA application changes the patterns of senescence. PAs delay senescence.	Capell <i>et al.</i> (1993)
PSII structure and electron transport	PA concentration affects the secondary structure and the rate of oxygen evolution of PSII. At very low concentration, they show a positive effect on photosynthetic activity and oxygen evolution, while at higher concentration, they reduce the rate of oxygen production. PAs inhibit photosynthetic electron transport in PSII.	Bograh <i>et al.</i> (1999)
Plant cell wall	PAs needed for cell wall maintenance and for strong links between cell wall components.	Berta <i>et al.</i> (1997)
Environmental stress	PA content changes when plants are stressed.	Kasukabe <i>et al.</i> (2004)
Drought Stress	Increased Spm and Spd confer drought tolerance.	Capell <i>et al.</i> (2004)
Cold Stress	Increased PA induction linked to cold tolerance.	Kasukabe <i>et al.</i> (2004)
Ozone sensitivity	Possible link between increase in PA and ozone tolerance.	Rowland-Bamford <i>et al.</i> (1989)
Plant Defence	PAs increase when plants are attacked by pathogens.	Walters (2003)
Osmotic stress	ADC2 is induced by osmotic stress.	Soyka <i>et al.</i> (1999)
Pollution Stress	Put accumulation an indicator of pollution stress.	Hauschild (1993)
Potassium deficiency	JA-induced putrescine accumulation occurs under potassium deficiency through the up-regulation of ADC2 but not ADC1 in <i>Arabidopsis</i> .	Armengaud <i>et al.</i> (2004)

### **1.10 Transgenic plants: holding clues to the regulation of polyamine metabolism**

In the charge to understand polyamine metabolism, transgenic plants have been created. By over- or under-expressing genes, researchers can examine what role these genes have in polyamine metabolism. Recently, researchers have used microarrays to investigate which other genes were altered in transgenic plants (Kasukabe *et al.* 2004). Table 2 shows the effects of changing the expression of some enzymes in polyamine metabolism. From these results, it is clear that polyamine metabolism is tightly regulated. Intriguingly, Rea *et al.* (2004) reported that a decrease in polyamine oxidases did not affect polyamine content, whereas other researchers have clearly shown that increases in ADC, SPDS and SAMDC can increase polyamine synthesis (Bassie *et al.* 2000). Bassie *et al.* (2000) showed that whilst mildly over-expressing polyamine synthesis genes does not cause changes in polyamines content, strong over-expression of polyamine synthesis genes can lead to abnormal phenotypes. Therefore, to use transgenics to examine the role of polyamines, researchers have to choose carefully which genes to change the expression of and control the extent to which expression is changed.



**Table 2. Effects of altered polyamine metabolism in transgenic plants.**

<b>Gene introduced</b>	<b>Effect</b>	<b>Plant</b>	<b>Ref.</b>
<i>SPDS</i> cDNA from <i>Cucurbita ficifolia</i> under CAMV 35S promoter	Increased Spd content in leaves. Increased stress tolerance. Increased expression of transcription factors such as DREB and stress-protective proteins like rd29A.	<i>Arabidopsis</i>	Kasukabe <i>et al.</i> 2004
Either maize polyamine oxidase (MPO) or pea copper amine oxidase (PCuAO)	No change in PA content, suggests either a tight regulation of PA content or a different compartmentalization of the two recombinant proteins.	Tobacco	Rea <i>et al.</i> 2004
Human <i>SAMDC</i> under CaMV 35S promoter	Put was decreased, Spd increased and Spm was increased or unchanged.	Tobacco	Noh <i>et al.</i> 1994
<i>Datura stramonium SAMDC</i>	Increase in Spd but not Spm in leaves and both Spd and Spm in seeds.	Rice	Capell <i>et al.</i> 2000
Yeast <i>SAMDC</i> with a ripening-inducible E8 promoter	Increased Spd, Spm, lycopene and ethylene.	Tomato	Mehta <i>et al.</i> 2002
Antisense or sense <i>SAMDC</i>	Increased <i>SAMDC</i> plants showed an increase in Spd and also an increase in the smaller numbers of tubers. Decreased <i>SAMDC</i> plants showed no phenotype.	Potato	Pedros <i>et al.</i> 1999
<i>ODC</i> cDNA from <i>Datura stramonium</i>	Increase in <i>ODC</i> activity far higher than increase in putrescine content. Again hints to a tight regulation of PA content.	Tobacco	Mayer <i>et al.</i> 2003
cDNA of Human <i>ODC</i>	No linear correlation between <i>ODC</i> mRNA, activity and PA accumulation suggesting PA regulation is more complex than in mammals. Regulation is different in different tissues.	Rice	Lepri <i>et al.</i> 2001
Antisense oat <i>ADC</i>	Decreased activity of both <i>ODC</i> and <i>ADC</i> causing a decrease in Put, Spd but not Spm.	Rice	Capell <i>et al.</i> 2000
<i>Datura stramonium ADC</i>	Higher concentration of PA synthesised under drought stress leading to tolerance.	Rice	Capell <i>et al.</i> 2004
Oat <i>ADC</i> under CaMV and constitutive maize ubiquitin 1 promoter	Promoter strength influences whether Spm or Spd are synthesised.	Rice	Bassie <i>et al.</i> 2000
Antisense oat <i>ADC</i>	Decreased <i>ADC</i> activity led to decreased Put and Spd. Rice <i>ODC</i> , <i>SAMDC</i> and <i>SPDS</i> expression unaffected.	Rice	Trung-Nghia <i>et al.</i> 2003
Antisense cDNAs for ACC synthase and ACC oxidase from carnation	Increased PA, causing increased seed production as well as stress tolerance.	Tobacco	Wi <i>et al.</i> 2002

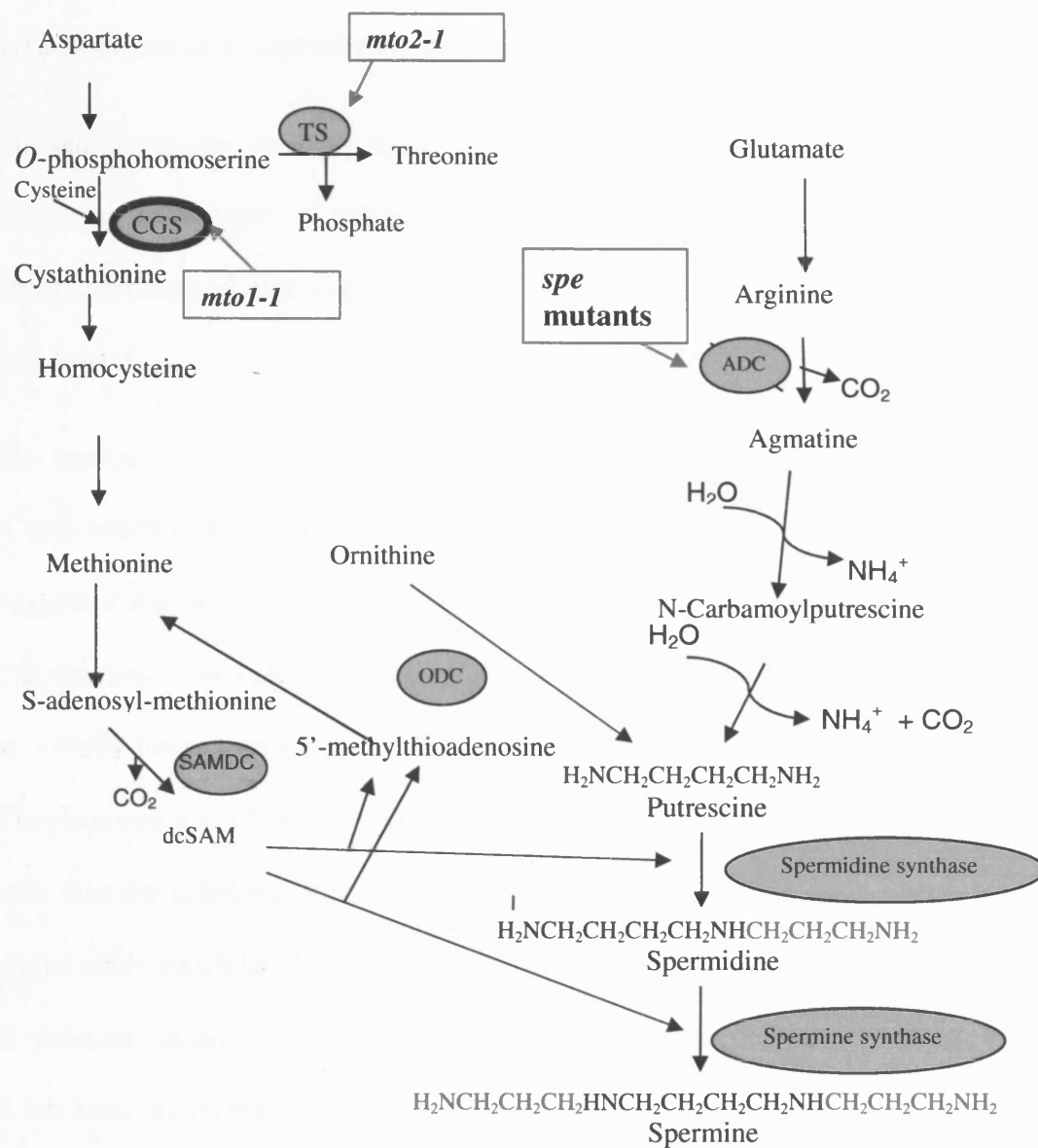
### 1.11 *Arabidopsis* polyamine synthesis/ response mutants

Several mutants in polyamine synthesis and response have already been found in *Arabidopsis* (Table 3). These have been created by a variety of methods.

**Table 3. Polyamine synthesis and response mutants in *Arabidopsis*.**

<b>Mutant</b>	<b>Effect</b>	<b>Ref</b>
<b>Polyamine synthesis mutants</b>		
<i>acl5</i>	Loss-of-function SPMS mutants show a severe defect in stem elongation	Hanzawa <i>et al.</i> 2002
<i>adc2-1</i>	Free Put content was reduced to about 25% of that in the control plants.	Urano <i>et al.</i> 2004
<i>spds1</i> and <i>spds2</i>	Spermidine synthase insertion T-DNA mutants. Double mutant seeds are shrunken.	Imai <i>et al.</i> 2004
<i>spe1-1</i> , <i>spe2-1</i>	ADC deficient mutants	Watson <i>et al.</i> 1998
<i>spms-1</i>	A T-DNA insertion mutant of the SPMS gene. Decreased free and conjugated spermine content.	Imai <i>et al.</i> 2004
<i>mtol-1</i>	Mutants show increased methionine production.	Inaba <i>et al.</i> 1994
<i>mtol2-1</i>	Mutants show increased methionine production.	Bartlem <i>et al.</i> 2000
<b>Polyamine response mutants</b>		
<i>AP2-10</i>	EMS mutant resistant to spermine but not Spd and Put, has developmental abnormalities.	Mirza <i>et al.</i> 1998
<i>par-1</i>	Enhancer tagged mutant screened for resistance to nor-spermidine. Lower uptake of spermidine.	Santiago, Poster at the 7 <sup>th</sup> International Conference of Plant Molecular Biology
<i>spr1-1</i>	EMS mutant resistant to spermine but not to Spd and Put, has abnormal leaves.	Mirza <i>et al.</i> 1997
<i>spr1-2</i>	EMS mutant resistant to spermine but not to Spd and Put, has abnormal leaves and flowers.	Mirza <i>et al.</i> 1997
35S::APETALA1	Spermidine unable to promote flowering in this mutant.	Applewhite <i>et al.</i> 2000
CS3123	Spermidine able to promote flowering in this late-flowering mutant.	Applewhite <i>et al.</i> 2000

From these mutants, experiments shall be conducted on the *spe* and *mtol* mutants.

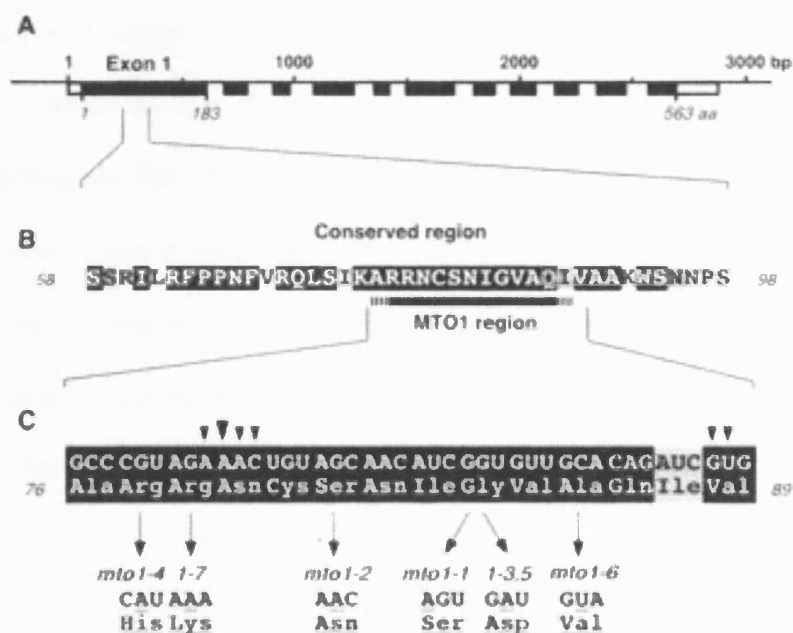


**Figure 3.** The polyamine pathway showing the *mto* mutants, increasing cystathionine-γ-synthase (CGS) activity and decreasing threonine synthase (TS) activity and the *spe* mutants decreasing ADC activity.

### 1.12 Methionine overproducing (*mto*) mutants

The *mto* mutations are likely to increase spermidine and spermine synthesis, as by over-producing methionine, decarboxylated S-adenosyl methionine, the aminopropyl donor for spermidine and spermine synthesis, is increased. Two mutants were studied, *mto1-1* and *mto2-1*.

The mutant *mto1-1* arose through a mutation in the first exon of cystathionine- $\gamma$ -synthase (CGS) which increases the stability of CGS mRNA. As early as 1985, Giovanelli *et al.* suggested that the regulation of CGS controls methionine synthesis. They postulated that the mechanism of control was an repression based system. More recently Chiba *et al.* (1999) backed up the theory of a repression mechanism regulated by methionine. They showed that CGS mRNA decreases in wild type Arabidopsis calli but not *mto1-1* calli that are subjected to exogenous methionine. There is a conserved region of 40 amino acids which has been suggested to be involved in post-transcriptional regulation. If mutations occur in this region, then methionine is overproduced (Figure 3, Figure 4). It has been suggested that the exon 1 polypeptide of CGS acts in cis to decrease its own RNA expression (Chiba *et al.* 1999). Although the mechanism of CGS regulation is unknown, it has been shown that the exogenous application of methionine and SAM can decrease CGS activity (Onouchi *et al.* 2004).



**Figure 4** The *mto1-1* mutation. From Onouchi *et al.* (2004).

CGS catalyses the production of methionine from *O*-phosphohomoserine (OPH), therefore the *mto1-1* mutant over-accumulates methionine 10-to 40-fold more than its wild type (Gakière *et al.* 2000). As plants flower, the methionine concentration in the rosette of *mto1-1* decreases to wild type levels, but in the inflorescence apex and in the fruits this mutant had 5-8 fold more methionine (Inaba *et al.* 1994). Inaba suggested that this was either due to the transport of methionine from the rosette to the inflorescence, or to methionine being degraded in the rosette and overproduced in the inflorescence. However, the decrease in methionine in the rosette during flowering is equal to the methionine increase in other tissues. It is therefore likely that excess methionine is exported out of the leaves. Either way, it would be interesting to see if excess methionine is converted to other products.

As threonine is also made from OPH, *mto1-1* has only 6% of the wild type soluble threonine. Furthermore, because methionine is a precursor of S-adenosylmethionine (SAM), the *mto1-1* mutant overproduces SAM 90-fold (Inaba *et al.* 1994). SAM is a precursor of both the ethylene and the polyamine pathway. Ethylene production in

*mtol-1* is only 40% higher than in the wild type (as ACC synthase, which forms ACC from SAM, catalyses the rate determining step for ethylene synthesis), so perhaps the polyamine pathway is also affected.

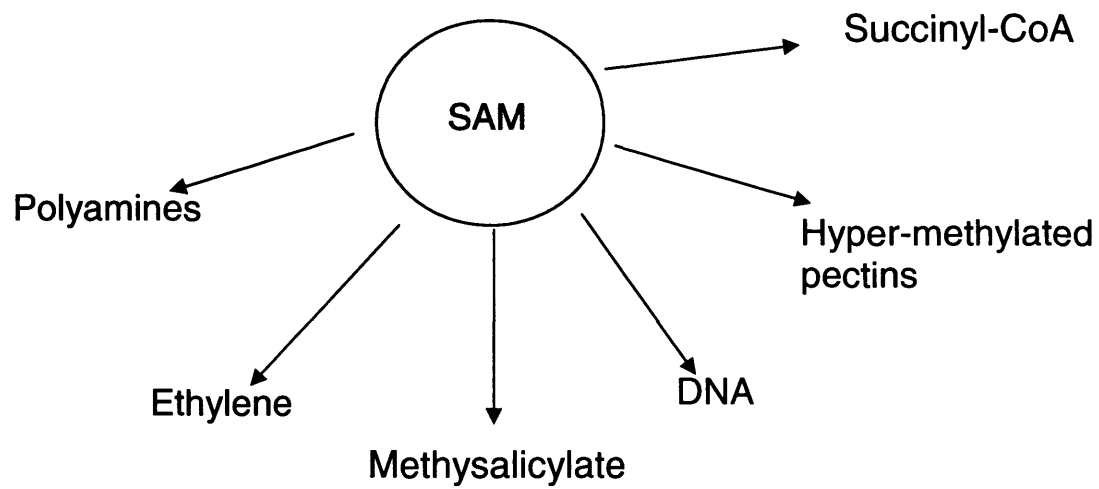
The mutant *mtol-1* has a mutation in the threonine synthase (TS) gene caused by a single base pair mutation, resulting in a leucine-204 to arginine change. Soluble threonine is reduced (Bartlem *et al.* 2000). This decreases competition from TS for OPH (O-phosphohomoserine) and therefore more OPH is converted to methionine. In *mtol-1* there is an increase in soluble methionine in the rosette by 20-fold compared to the wild type (Ws-2). Like *mtol-1*, in *mtol-1* SAM is overproduced, up to 3-fold more than Ws-2 (Bartlem *et al.* 2000). The concentrations of soluble Asp, Lys, and Ile did not change, hinting that *mtol-1* does not differ in the steps after or before Thr formation when compared to the wild type.

Unlike *mtol-1*, the *mtol-1* mutation had an effect on plant physiology (Bartlem *et al.* 2000). Although germination was similar between the *mtol-1* mutant and the wild type, a greater number of *mtol-1* seedlings appeared to stall after germination and did not recover. Furthermore, cotyledon opening was delayed in *mtol-1*, the average fresh weight of the *mtol-1* tissues was between 30% and 50% of the wild type and the average length of *mtol-1* roots at day 8 was 22% of the wild type seedlings. Polyamine content has not been studied in the *mtol-1* mutant. Interestingly the changes in methionine accumulation in both *mtol* mutants are affected by plant growth and on entering the reproductive cycle, but the accumulation of methionine is always higher than in the wild type (Bartlem *et al.* 2000, Inaba *et al.* 1994).

Although SAM is over-produced 90-fold in the *Arabidopsis mto1-1* mutant, ethylene production in the *mtol-1* mutant only increases by 40%. This suggests that the excess SAM seen in the *mtol* mutants may lead to an increase in spermidine and perhaps

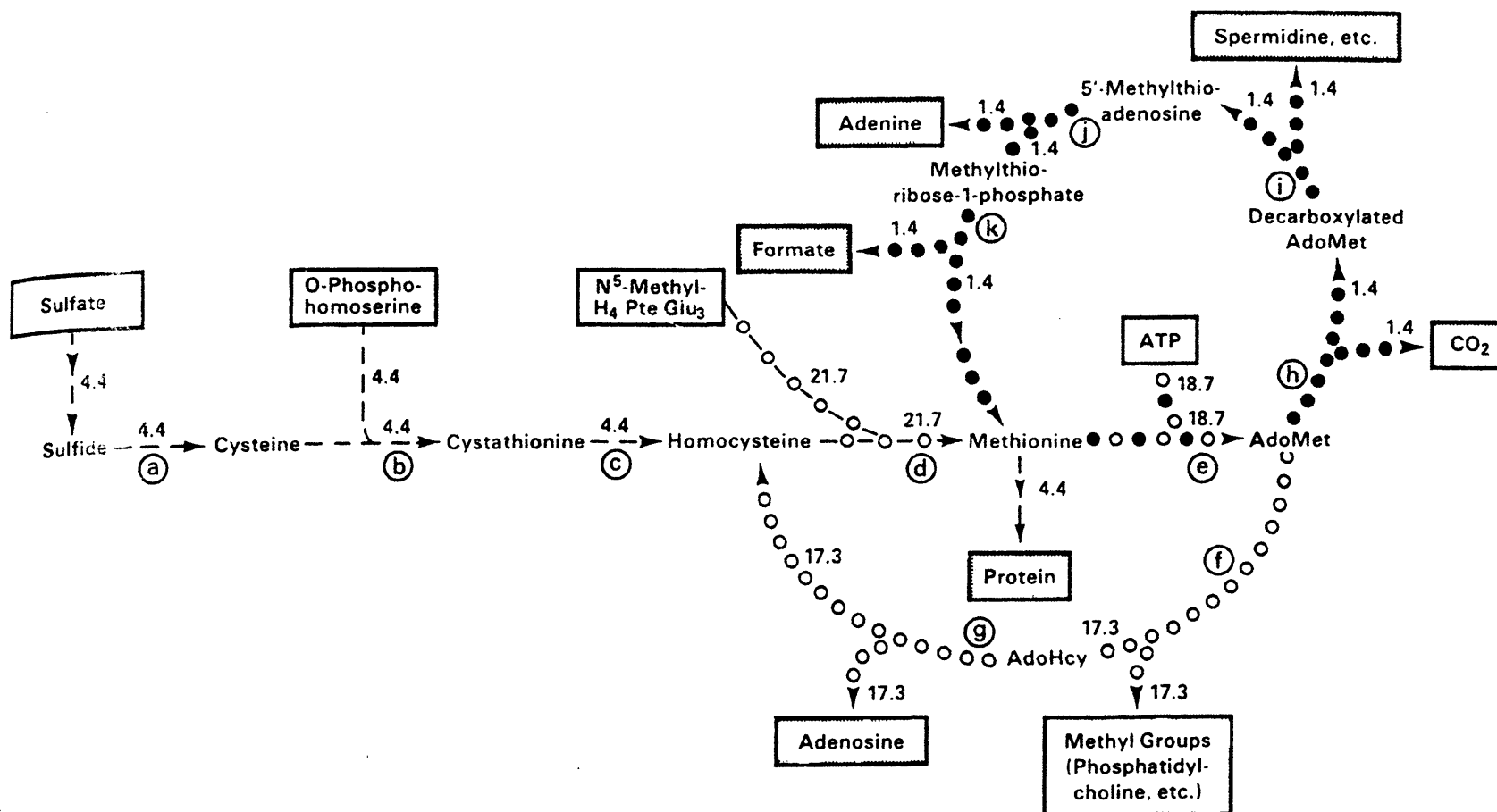
spermine. Therefore, it is expected that polyamine content would change in these mutants. Spermidine production has been shown to correlate with the tissue-specific regulation of SAMDC and SPDS in plants (Malmberg *et al.* 1998). SAMDC has been postulated to be the rate determining step for spermidine synthesis under normal conditions in *Arabidopsis* (Tassoni *et al.* 2000), yet transgenic tobacco plants with increased SAMDC activity and dcSAM accumulation showed no change in spermidine levels (Hanfrey *et al.* 2002). This indicates that the regulation of polyamine biosynthesis is complex and not completely defined. It is therefore unclear whether the *mto* mutants will show increased polyamine synthesis. Whether the *mto* mutants show a different polyamine content than Col-0 under stress is dependent on how polyamine formation is regulated. Figure 2 shows that the *ADC2* gene is up-regulated under salt and osmotic stress. This could change the level of putrescine available for the synthesis of spermidine and spermine. As *SAMDC* expression does not increase, control exerted by SAMDC over the pathways for spermidine and spermine synthesis may increase under stress conditions. Assuming that SAMDC does not operate at substrate saturation, increased availability of SAM in the *mto* mutants may thus enhance flux through the spermidine/spermine synthesis pathway.

It is not only the polyamine pathway that may be affected by the *mto* mutations. Figure 6 and Figure 5 shows the major pathways for methionine metabolism and indicates how excess methionine may be metabolised. Furthermore, in tomato, mutants with increased SAMDC had increased ethylene synthesis as well as increased polyamine content (Mehta *et al.* 2002). This is as the polyamine and ethylene pathways are linked through SAM. Excess SAM could also bind to DNA or be involved in the creation of hyper-methylated pectin and methylsalicylate (Ross *et al.* 1999). SAM can also be degraded by a pathway resulting in the production of succinyl-CoA (Figure 5).



**Figure 5** Where does all the SAM go?





v

**Figure 6.** The major pathways for methionine metabolism. Enzymes are as follows: a, cysteine synthase; b, cystathionine  $\gamma$ -synthase; c, cystathionine  $\beta$ -lyase; d, tetrahydropteroyltriglutamate methyltransferase; e, methionine adenosyl-transferase; g, AdoHcy hydrolase; h, AdoMet decarboxylase; i, spermidine synthase. Open circles represent transmethylation and closed circles represent methylthio recycling, whilst dashes represent the pathway for the synthesis of methionine. Flux numbers are indicated. Adapted from Giovannelli *et al.* (1985).

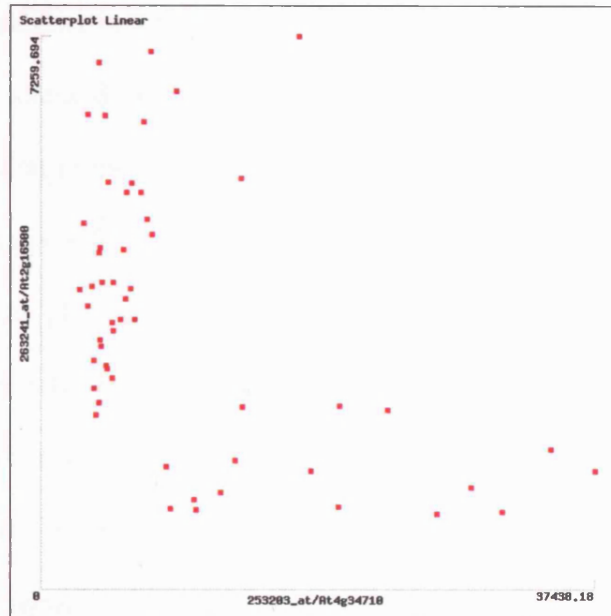
### 1.13 *spe* mutants

The *spe* mutants were isolated by screening of 15,000 EMS mutagenised M<sub>2</sub> seedlings for low ADC activity (Watson *et al.* 1998). *spe2-1* mapped to *ADC2* and Malmberg (1998) postulated that *spe1-1* is a mutant in the *ADC1* gene, although this mutant is not mapped. The *spe1-1* mutant has 23% ADC activity and *spe2-1* has 36% ADC activity. These mutants differ from their wild type by an increase in lateral root initiation and growth. When the double mutant *spe1-1/spe2-1* was created, the ADC activity dropped to 18% and root growth was severely kinked with a compact pattern, the leaves narrowed and flowering was delayed by one week. This response suggests that the effect of ADC activity is dose-dependent. However, although it has been postulated that *spe1-1* maps to *ADC1* this has not been proved. Instead, *spe1-1* may be a mutant in the transcriptional regulation of both or one of ADC genes.

In *spe1-1* and *spe2-1*, putrescine formation will be affected by the decrease in ADC activity. This may lead to other polyamines being decreased too, as a knock-on effect. Capell (2004) postulated that a certain concentration of putrescine in the pool needed to be created before spermidine and spermine content was increased and showed that increased ADC expression in transgenic rice led to increased drought tolerance. If this theory is true, then the *spe* mutants, with their decreased ADC activity, will take longer to increase the putrescine content to above this critical threshold level and therefore be more sensitive to stress.

As *ADC2* is upregulated in stress conditions (Urano *et al.* 2003), there is a possibility that the *spe2-1* mutant, which is deficient in *ADC2* activity, may be more sensitive to stress than its wild type.

Genevestigator (<https://www.genevestigator.ethz.ch/>) was used to compare the levels of *ADC1* and *ADC2* transcripts under different stress conditions.



**Figure 7.** Relationship between levels of *ADC2* (y axis) and *ADC1* (x axis) transcripts in plants grown under normal conditions and under salt, osmotic or drought stress. The figure was produced by analysing Affymetrix GeneChip data from the AtGenExpress experiments using Genevestigator (<https://www.genevestigator.ethz.ch/>).

Figure 7 shows that the two *Arabidopsis* *ADC* genes are not both highly expressed at the same time in plants grown under normal conditions or under stress conditions. This indicates that *ADC1* and *ADC2* have distinct functions. As *ADC1* is not regulated in the same way as *ADC2*, it shall be interesting to see how the *spe1-1* and *spe2-1* mutants differ under stress conditions.

### 1.14 Salt stress

In this thesis, the effect of changes in the polyamine pathway on salt tolerance was examined. This was done by examining the growth of the *mto* and *spe* mutants on salt-supplemented medium.

The increased soil salinity in the twentieth century has had an increasing impact on crop productivity. Salt stress decreases germination, tissue mass and yield (Zapata *et al.* 2003). This means that saline soils are considered disadvantageous for plant growth.

Salinity has two major effects, ionic and osmotic stress. Ionic stress results from the salt accumulation in plant cells and osmotic is caused by salt present in the soil. Osmotic stress is caused due to the water potential imbalance between the apoplast and symplast. This makes it difficult for plants to absorb nutrients and water. Saline soils increase the external osmotic potential, causing cell dehydration which can affect membranes and proteins and causes the accumulation of toxic ions (ionic stress), namely  $\text{Na}^+$  and  $\text{Cl}^-$ . The effect of stress on the cellular level is reflected in the overall well-being of the plant. If salt stress is severe, it can lead to a decrease in cell turgor, which affects plant growth. As  $\text{Na}^+$  and  $\text{Cl}^-$  accumulate in leaves, this causes wilting and in extreme cases cell death occurs (Lu *et al.* 2002). The accumulation of salt affects the ionic balance in the cell. For example it can reduce the  $\text{K}^+/\text{Na}^+$  ratio, which is important for maintaining metabolic processes (Ashraf and Khanum, 1997).

Plants have many mechanisms to deal with salinity. They can compartmentalize toxic ions into vesicles, such as the vacuole, or into older leaves. Movement of ions into the vacuole of a healthy cell must not disturb the makeup of the cytosol. To minimise disturbance to the homeostatic state of the cell, ions are transported through vesicles or a cytological process that juxtaposes the plasma membrane to the tonoplast (Hasegawa *et al.* 2000) or by simultaneously transporting ions into the cell and vacuole at the same

rate (Yokoi *et al.* 2002, Xiong *et al.* 2002). Alternatively, plants can exclude salt from their roots via transport proteins (Zhu 2003), excrete salt on to the surface of leaves via glands or bladders or have a higher tolerance to increased salt. This tolerance is based on a variety of responses including adjustment of osmolytes. Polyamines belong to a group of lower mass osmolytes such as ectoine, glycine betaine, trehalose and proline (Jantaro *et al.* 2003). Osmoprotectants have been said to have several roles, from the scavenging of reactive oxygen species (Bohnert and Jensen 1996) to aiding the maintenance of a favourable osmotic potential between the cell and its surroundings (Pollard and Wyn Jones, 1979).

The role of polyamines in stress tolerance has remained elusive. Polyamines have been reported to increase or decrease under salt stress, dependent on the time of evaluation, species studied and type of stress imposed. Whilst some researchers observed that salinity decreases polyamine content (Prakash *et al.* 1988), some maintain that salt stress increases polyamine content (Chattopadhyay *et al.* 1997). Others report that individual polyamines may increase whilst others decrease. Zapata *et al.* (2003) reported that salt-treatment reduced the putrescine level, while spermidine and spermine increased in lettuce. Work that involves the pre-treatment with polyamines showed a reduction in the deleterious effects of NaCl (Mansour *et al.* 1993). Again, published data is inconsistent as pre-treatment experiments with precursors of putrescine biosynthesis did not decrease salt stress in rice seedlings (Lin and Kao 1995). Table 4 and Table 5 show a summary of some recent publications.

**Table 4. Reports of the positive effect of polyamines on salt tolerance.**

<b>PA increase/decrease</b>	<b>Other effect</b>	<b>Species</b>	<b>Reference</b>
Increased Spm and Spd	Cellular alterations caused by NaCl in inner epidermis reduced.	Onion	Mansour <i>et al.</i> 1998
Increased free and conjugated Pas	Heat stress increased PA in tolerant plants not in sensitive plants.	Rice	Roy <i>et al.</i> 2001
Increased Put	Sorbitol induced ADC and Put in leaves. No experiments on tolerance.	<i>Arabidopsis</i>	Feirer <i>et al.</i> 1998
Increased Pas	Pre-treatment with PA decreased lipid peroxidation caused by oxidative stress.	Sunflower	Groppa <i>et al.</i> 2001
Increased Pas	Decreased ethylene genes, increased PA production and decreased chlorophyll loss after oxidative, salt and acid stress.	Tobacco	Wi <i>et al.</i> 2002
Increased Put	Increased Put in salt tolerant plants compared to salt sensitive plants.	Tomato	Santa Cruz <i>et al.</i> 1997
Increased Spd and Put	Increased tolerance to salt and drought stress.	Tobacco	Waie <i>et al.</i> 2003
Increased Spd	Increased spermidine synthase activity creating higher spermidine content showed resistance to high salinity.	<i>Arabidopsis</i>	Kasukabe <i>et al.</i> 2004
Increased Put	If Put is decreased. salt tolerance also decreases.	<i>Arabidopsis</i>	Urano <i>et al.</i> 2004

**Table 5 Reports of the negative/null effects of polyamines on salt tolerance.**

<b>PA increase/decrease</b>	<b>Other effects</b>	<b>Species</b>	<b>Reference</b>
No effect	Comparison of PA levels and salt tolerance.	Rice	Lefevre <i>et al.</i> 2001
No effect	Comparison of PAs and germination under salinity.	Lettuce	Zapata <i>et al.</i> 2003
No effect	Fe stress does not induce PA.	Tobacco	Lovaas <i>et al.</i> 1997
Increased Spd and Put	Salinity increased Spm more in sensitive than tolerant plants.	Wheat	Galiba <i>et al.</i> 1993
Put and Spm increased, Spd decreased	In saline conditions, no physiological effect on plant monitored.	<i>Arabidopsis</i>	Urano <i>et al.</i> 2003

### **1.15 Isolating *Arabidopsis* polyamine response mutants**

Mirza *et al.* (1998) showed that by screening plants on polyamine-supplemented media, polyamine response mutants could be isolated. In this thesis, activation-tagged *Arabidopsis* lines were screened on spermidine-supplemented medium to obtain mutants.

When *Arabidopsis* was subjected to spermidine at concentrations of 0.5 mM, treated plants grew with darker green leaves, shorter stalks and root growth was decreased while chlorophyll production was increased (Tassoni *et al.* 2000). At higher concentrations, polyamines are more toxic. Mirza *et al.* (1998) experimented with *Arabidopsis* growth media with spermidine levels of 1-8 mM. They showed that root growth and germination decreased, and at 8 mM no plants germinated. Tassoni *et al.* (2000) reported that spermidine-treated *Arabidopsis* cotyledons can accumulate spermidine in concentrations of 4.6 mM and that excess spermidine can be converted into putrescine. They also proved that the toxicity of polyamines is not related to their

charge. Spermidine can be metabolized to hydrogen peroxide ( $H_2O_2$ ) and ammonium, which are toxic to cells and can cause cell damage (Seiler 1995). Spermidine and spermine can induce bleaching of barley leaves (Srivastava 1987). As  $H_2O_2$  builds up, the plant cannot degrade all of it into water and oxygen and this eventually results in the bleaching of plants. In fact, mutants which were made to over-produce polyamines show a variety of defects including decreased root length, thinning of leaves and stems, chlorosis and necrosis (Hanzawa *et al.* 2000). The production of  $H_2O_2$  through polyamine oxidation has also been correlated with the oxidative burst and cell death (Allan and Fluhr 1997, Møller and McPherson 1998).

There are a variety of different metabolic and signalling pathways that could be affected in spermidine-resistant mutants. In this thesis, plants were screened for long roots or delayed bleaching when grown on spermidine. Mutants that are resistant to spermidine-supplemented medium could have a mutation in the signalling, storage, transport, synthesis or catabolism of polyamines. Resistance could be due to the enhancement or disruption of a single protein activity, or a change in gene expression of many genes caused by altered expression of a transcription factor. It could be due to a mutation that stopped spermidine being transported into the plant, e.g. due to the knockout of a transport protein or enhancement of an efflux mechanism. Alternatively, excess spermidine could be stored in vesicles or in the vacuole. Again this would involve transporters, or perhaps the enhancement of a storage mechanism. If the catabolism of  $H_2O_2$  is enhanced, this would prevent the toxic effects of spermidine breakdown. Polyamines are interconvertible, so excess spermidine may be channelled into spermine or putrescine. Another possibility is that a biosynthetic enzyme could be knocked out leading to a requirement for spermidine in the medium.



However, spermidine resistance may also be caused by a mutation that is not directly related to spermidine. In a root length mutant, it could be that a protein that enhances general root growth is affected. In a stay-green mutant, it could be that chlorophyll is overproduced or not degraded efficiently.

In short, there are many genes that can be changed and the only way to know for sure is to obtain the DNA sequence that has been affected. Once the mutation is identified, it is possible to compare the phenotype of the mutant with its wild type in order to obtain more information on the function of polyamine-related compounds in plants.

### **1.16 Aims of the thesis**

The two main aims of this thesis were:

To test the reaction of *Arabidopsis* polyamine synthesis mutants to salt and osmotic stress. To investigate the reaction of wild type and mutant plants to both stresses, evaluate the different responses and look closer at the role of polyamines in stress tolerance.

To screen activation-tagged lines in order to obtain mutants resistant to spermidine. To re-screen these plants, make backcrosses and analyse the segregation patterns. To find the genomic location of the mutation.

## **Chapter Two: Determination of polyamine contents in wild type plants and in the *mta* and *spe* mutants**

## **2.1 Aims**

The main aims of this chapter were to optimise the method for polyamine extraction and quantification in *Arabidopsis* and to determine polyamine contents in different organs of wild type *Arabidopsis* and in the *mto* and *spe* mutants. To this end, recovery of polyamines from *Arabidopsis* tissue was determined and standard curves were obtained.

## **2.2 Introduction**

### **2.2.1 Methods of polyamine determination**

Currently there are two ways researchers separate plant polyamines, through HPLC (Hummel *et al.* 2002) and thin-layer chromatography (TLC). Both systems rely on the separation of polyamines in an organic solvent. To visualise polyamines, they are labelled. For U.V. visualisation, dansyl chloride can be used. Dansylated amines can be quantified through use of a fluorometer. An excitation wavelength of 365 nm and an emission wavelength of 510 nm can be used.

HPLC advantages:

- More accurate
- Detection of conjugated and non-conjugated polyamines

TLC advantages:

- Cheap
- Easy methodology

In this thesis, TLC was used to separate and quantify polyamines. TLC allows polyamines to be separated by their solvency in a mix of chloroform:triethylamine (4:1).

Bands were visualised under U.V. and cut out. Polyamines were eluted and quantified using a spectrofluorometer.

The table below gives the polyamine content in plants from other research groups. *Arabidopsis* polyamine data was determined from HPLC methods and used fresh plant material, apart from Hummel *et al.* (2004) who used dry plant material. Note that if data was given in fresh weight, values were multiplied by ten to get contents on a dry weight basis. Variation in polyamine content can be explained by differences in ecotype, method of growth and time of sampling.

**Table 6** Polyamine levels in different organisms in  $\mu\text{mol/g DW}$

Spermine Level	Spermidine Level	Putrescine Level	Organism	Reference
0.2-0.4	1.2-1.4	0.5-1.5	<i>Arabidopsis</i>	Hummel <i>et al.</i> 2004
0.3-0.2	2-3	0.50-2	<i>Arabidopsis</i>	Urano <i>et al.</i> 2004
0.5-1.5	3.4-4.5	0.5-1.2	<i>Arabidopsis</i>	Imai <i>et al.</i> 2004
0.2-1.5	1.5-20	0.2-5	<i>Arabidopsis</i> (untreated plants and plants treated with 0.5mM SPD)	Tassoni <i>et al.</i> 2000
0.1-0.3	0.8-1.2	5-22	<i>Pringlea antiscorbutica</i>	Hummel <i>et al.</i> 2004
4	11	94	<i>Rannunculus</i>	Chang <i>et al.</i> 1999
1.24 – 2.3	8.49 – 26	2.84 – 6.75	Soyabean	Kang <i>et al.</i> 1998
5-90	5 – 90	5 – 35	Sunflower (treated with exogenous polyamine)	Benavides <i>et al.</i> 2000

## 2.3 Materials and Methods

### 2.3.1 Plant material

Leaf tissue from *Arabidopsis thaliana* L. (Columbia-0) was used to optimise extraction methods. Seed was obtained from the Nottingham *Arabidopsis* Stock Centre (NASC).

After the extraction method was optimised, the following mutants and their respective wild types were analysed for changes in polyamine contents (Table 7). All plants were sown onto soil and grown a 12-h light:12-h dark regime and 50% relative humidity at 22°C in the day and 18°C at night. Light intensity was approximately 100  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . After plants had flowered, material was harvested from leaf, stem and flowers for determination of the polyamine content.

**Table 7.** Mutants analysed for changes in polyamine content.

Mutant (Background) [NASC Stock number]	Phenotype
<i>mtol1-1</i> (Col-0) [N196]	Over expressed CGS (cystathionine- $\gamma$ -synthase) mRNA, thus increased methionine production (Naito <i>et al.</i> 1994).
<i>mtol2-1</i> (Ws-2) [N208]	Pathway from OPH (O-phosphohomoserine) to Thr blocked therefore more OPH converted to methionine due to less competition for substrate (Bartlem <i>et al.</i> 2000).
<i>spe1-1</i> (Col-0) [N3766]	ADC activity 23-36% (Watson <i>et al.</i> 1998).
<i>spe2-1</i> (Ler-0) [N3767]	ADC activity 39-50% (Watson <i>et al.</i> 1998).

### 2.3.2 Polyamine analysis

Polyamines were extracted following a method similar to De Agazio *et al.* (1995) except that toluene was used instead of ethyl acetate for extraction and a 3 min centrifugation step was introduced after adding toluene, which increased recovery rates. Each *Arabidopsis* plant was frozen immediately in liquid nitrogen and then freeze-dried. *Arabidopsis* tissue was ground and free polyamines were extracted in 1200  $\mu\text{l}$  of 10 % (w/v) cold perchloric acid (PCA) and then centrifuged at  $13000 \times g$  for 15 min at 4 °C. 125  $\mu\text{l}$  of supernatant was added to 250  $\mu\text{l}$  saturated sodium carbonate solution and 250  $\mu\text{l}$  dansyl chloride (5 mg  $\text{ml}^{-1}$ ). Dansylation occurred in darkness at room temperature, overnight. Dansylated polyamines were extracted by addition of 312  $\mu\text{l}$  toluene and 62.5  $\mu\text{l}$  of L-proline (100 mg  $\text{ml}^{-1}$ ). Samples were centrifuged for 3 min and 50  $\mu\text{l}$  of each sample was spotted onto a thin layer chromatography plate (pre-coated with 0.25 mm silica gel with fluorescent indicator F-254; Merck, Darmstadt, Germany) and run in a solvent of chloroform:triethylamine (4:1). Polyamines were visualised by U.V. Bands were cut out and polyamines extracted in 1 ml of acetone. Polyamines were quantified at 505 nm (emission) and 345 nm (excitation) using the Perkin-Elmer LS50 luminescence spectrometer (Perkin-Elmer, Gaithersburg, Maryland, USA). Standard curves were used to calculate the polyamine content in the *Arabidopsis* extracts. In addition, a polyamine standard was analysed in each experiment to ensure that signal values did not change between different experiments.

### 2.3.2 Polyamine analysis

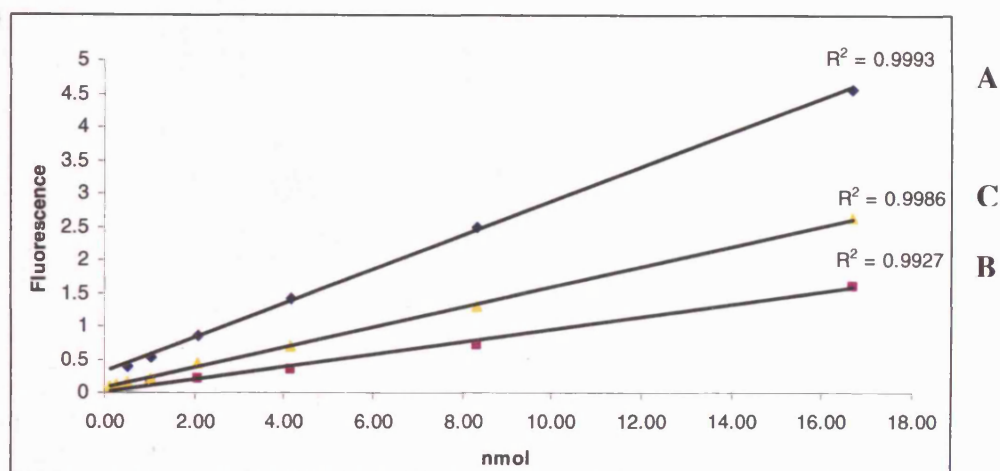
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## 2.4 Results

### 2.4.1 Standard curves for polyamine determination

Firstly, a standard curve was created for each of the polyamines (Figure 8). The fluorescence signal determined after extraction of the dansylated polyamines from TLC plates showed a linear increase with increasing amounts of each polyamine.



**Figure 8.** Standard curves for A. spermine, B. spermidine and C. putrescine.

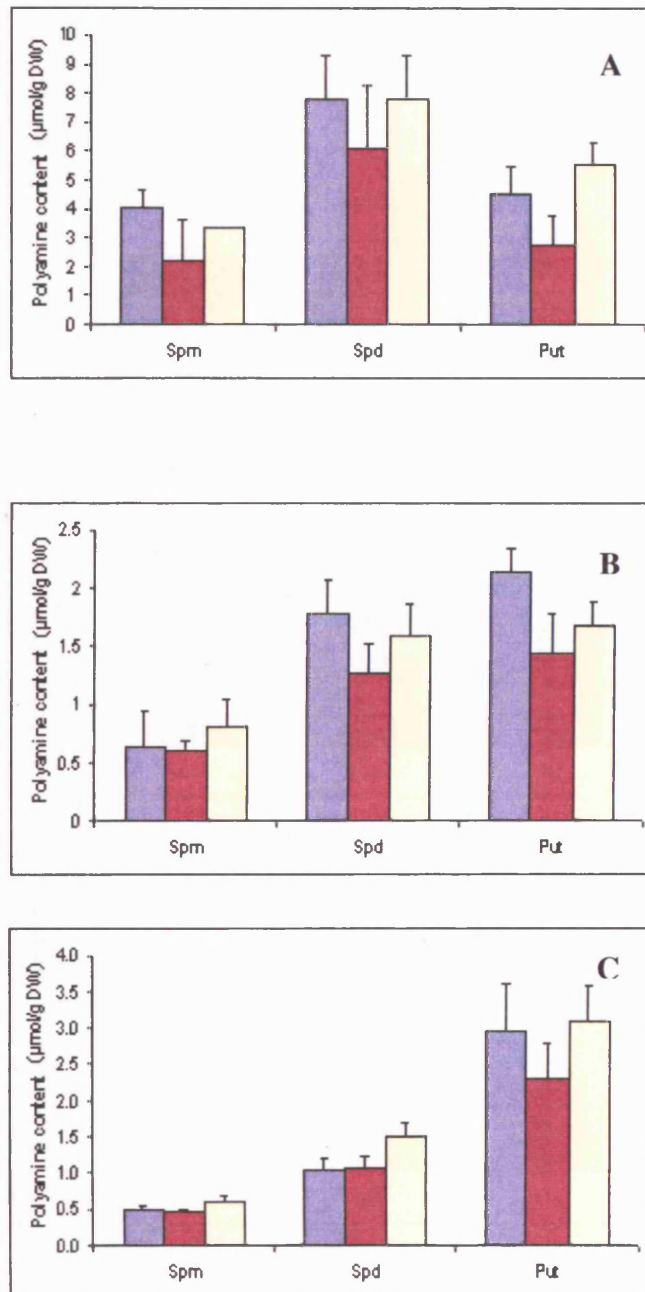
To determine whether the extraction procedure had an adverse affect on the recovery of polyamines, a known amount of each polyamine was added to 0.2 g of plant leaf material. Initially, recovery from plant material mixed with known concentrations of polyamines was low. By replacing ethyl acetate with toluene and including a 3 min centrifugation step, recovery was boosted to between 85-100% (Table 8).

**Table 8.** Recovery of polyamines from plant material.

	Fluorescence readings	
	Ethyl acetate	Toulene
Spm + Spd + Put (200 nmol)	50	44
Leaf	30	14
Leaf + Spm + Spd + Put (200 nmol)	49	60
<b>% Recovery</b>	<b>60%</b>	<b>103%</b>

### 2.4.2 Polyamine content in Columbia, *mtol-1* and *spe1-1*

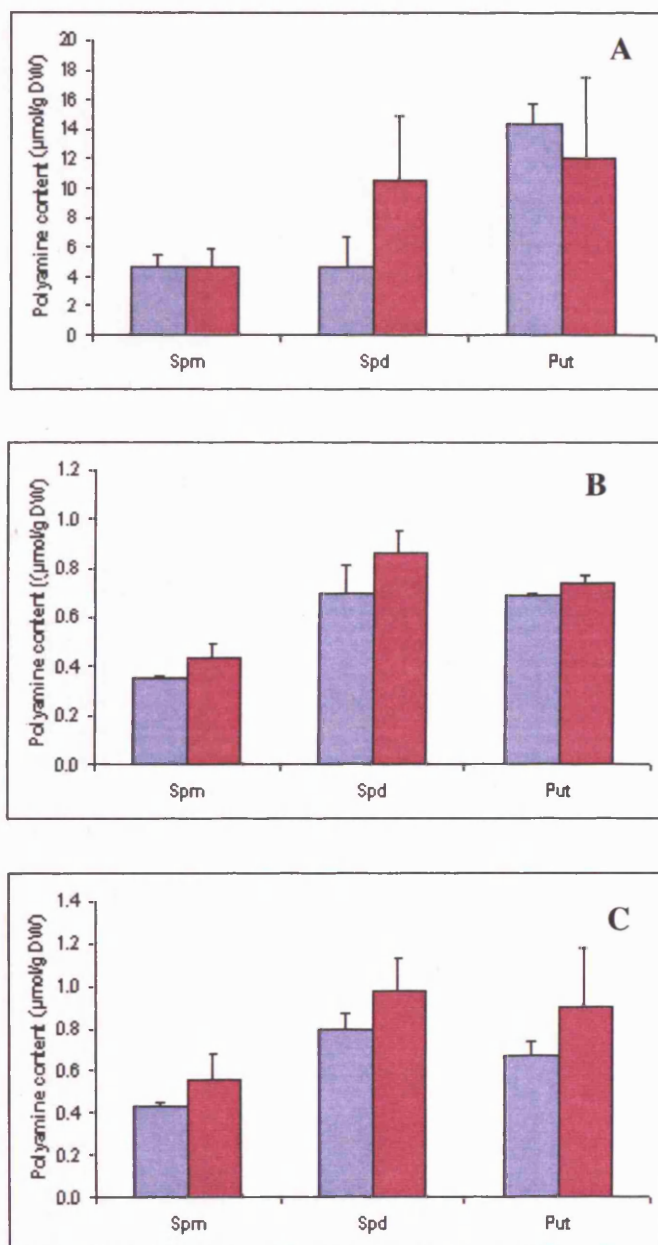
There seemed to be no difference in polyamine content between the mutants and wild type (Figure 9). Mutants and wild type plants contained more polyamine in the flowers than in the leaves or stems.



**Figure 9.** Polyamine content in flower (A), leaf (B), stem (C) tissue of *mtol-1* (red), *spe1-1* (yellow) and Col-0 (blue). Values are means of 5 plants. Error bars represent standard error.

### 2.4.3 Ws-2 and *mto2-1*

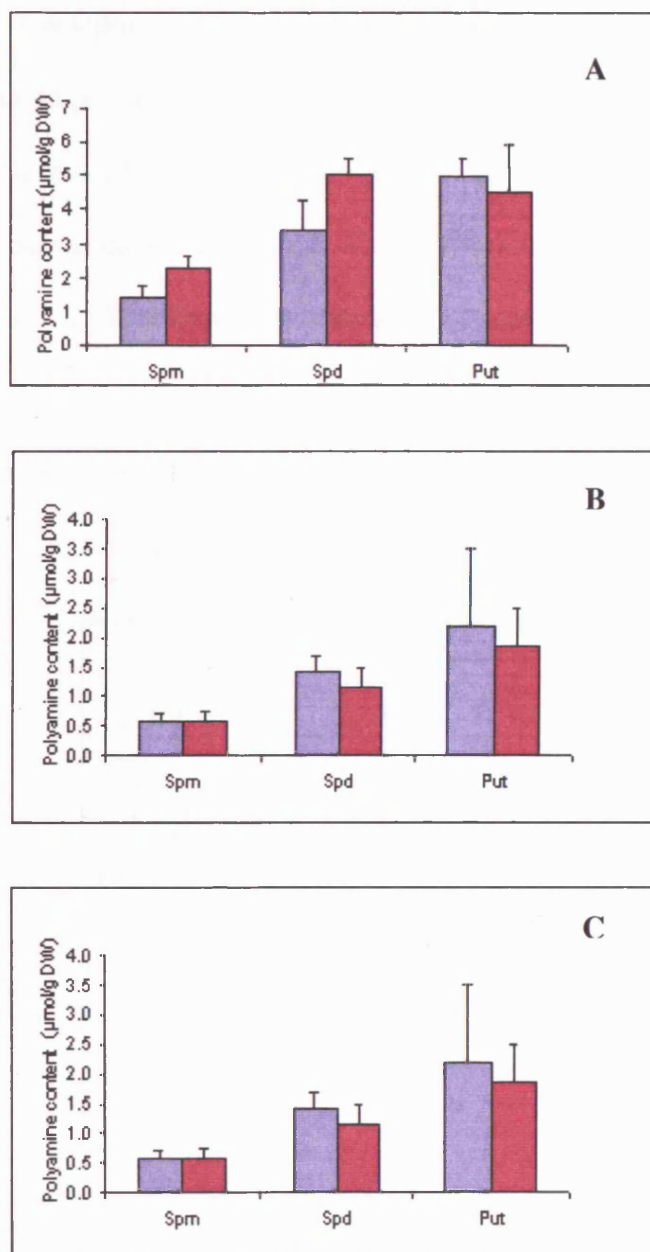
Again, the flowers showed a higher polyamine content than leaf and stem tissue. Under normal conditions, the extra methionine produced in the *mto2-1* mutant did not lead to the accumulation of spermidine or spermine (Figure 10).



**Figure 10.** Polyamine content in flower (A), leaf (B), stem (C) tissue in *mto2-1* (red) and Ws-2 (blue). Values are means of 5 plants. Error bars represent standard error.

#### 2.4.4 Ler and *spe2-1*

Again, spermine and spermidine were higher in the flower tissue than the green tissue (Figure 11). Putrescine content was higher in the leaf and flower than in the stem tissue. There was no difference between the *spe2-1* mutant and its wild type.



**Figure 11.** Polyamine content in flower (A), leaf (B), stem (C) in *spe2-1* (red) and Ler-0 (blue). Values are means of 5 plants. Error bars represent standard error.

#### 2.4.5 Analysis of (Spm+Spd)/Put Ratio

Table 1 shows the (Spm+Spd)/Put ratio determined from the average polyamine content. These are shown to show the change in the levels of putrescine compared to its derivatives spermine and spermidine. The (Spm+Spd)/Put ratio has been shown to correlate with the developmental stage of the embryo (Minocha *et al.* 2004). However, is there a difference in polyamine ratio between the tissues of plants? Apart from Ws-2 and *mto2-1* the ratio was higher in the flower than in either stem or leaf tissue. This is as the increase in putrescine content in the Ws-2 flowers was more than the increase in spermine or spermidine. Therefore, the ratio does not seem to follow a trend across the different lines.

**Table 9.** The (Spm+Spd)/Put ratio using values in  $\mu\text{mol/g DW}$ .

(Spm+Spd)/Put			
	Flower	Stem	Leaf
Col	2.63	0.52	1.13
<i>mto1-1</i>	3.01	0.67	1.29
<i>spe1-1</i>	2.03	0.67	1.4
Ler	0.98	0.92	0.40
<i>spe2-1</i>	1.63	0.93	0.56
Ws-2	0.65	1.53	1.84
<i>mto2-1</i>	1.26	1.76	1.69

## **2.5 Discussion**

### **2.5.1 Polyamine determination**

Recovery of polyamines from *Arabidopsis* was higher when extraction was carried out with toluene rather than ethyl acetate. Standard curves showed that at these concentrations, there was a linear relationship between the amount of polyamine and fluorescence.

### **2.5.2 Polyamine content in *Arabidopsis***

In general, in *Arabidopsis*, flowers had a higher polyamine content than green matter. Polyamines have been shown to accumulate in sexual organs before by Urano *et al.* (2003). Applewhite (2000) also showed that polyamine contents were higher in Columbia flowers with spermidine accumulating more than putrescine. The (Spm+Spd)/Put ratio observed in these experiments also indicates that, similar to Applewhite, putrescine accumulation in the flowers is lower than accumulation of spermidine and spermine. In contrast, in *Sinapsis* it was shown that putrescine was transported from mature leaves at flowering (Havelange *et al.* 1996). Perhaps spermidine and putrescine have similar roles in the flower and it does not matter which of these two polyamines accumulates or, if polyamines can be interconverted, it may not matter which polyamine is initially transported to the flower.

Interestingly, Thu-Hang *et al.* (2002) linked the variation of polyamine content between tissues to their metabolic activity; the lower the metabolic, rate the higher the level of polyamines. Furthermore, polyamines could accumulate to provoke flowering, not as a result. This theory is supported by the fact that polyamines are important in cell division as the rates of cell division are increased in flowering tissues (Cohen 1998). Another theory is that polyamines are detrimental to vegetative growth. This is

supported by Masgrau *et al.* (1997), who showed that putrescine is toxic in vegetative plants of tobacco but not in flowering plants.

### **2.5.3 *spe* mutants**

No difference was found in the polyamine content of the leaf, stem and flower between the *spe* mutants and the wild type; however, the root tissue was not examined. Root polyamine content was determined by Watson *et al.* (1998), who showed that there was no difference in polyamine content in the root tissue of the mutants and their respective wild types. They did, however, observe a reduced root polyamine content in the double mutant *spe1-1/spe2-1*.

### **2.5.4 *mto* mutants**

The *mtol-1* mutant showed no increase in spermidine or spermine content in any of the green tissue material or flowers compared to Col-0, even though Naito *et al.* 1994 have shown there is an increase in methionine in *mtol-1*. Naito *et al.* (1994) reported that in *mtol-1*, with the onset of reproductive growth, methionine accumulation switches from the rosette to the inflorescence apex. The *mtol-1* mutant had a higher soluble methionine content in the inflorescence apex than Col-0. Yet, these results show that this higher floral methionine content is not reflected in the polyamine content. Similarly, no difference in polyamine content was seen between *mtol-2* and Ws-2. Franceschetti *et al.* (2004) also observed in transgenic tobacco with increased SPDS that there was no change in polyamine content. They postulated that polyamine synthesis is tightly regulated and to get an increase ODC, ADC and SAMDC would have to be enhanced. The increase in SAM in the *mtol* mutants indicates that steps before the SAMDC reaction do not limit the polyamine synthesis under normal conditions. SAM

feeds into both the polyamine and ethylene pathways as well as many transmethylation reactions (e.g. proteins, phospholipids, DNA and RNA). Excess SAM, therefore, can either build up or be channelled into any one of these routes (see explanation on page 35-36).

### **2.5.5 Control of polyamine synthesis in plants**

In animal cells, increased spermine or spermidine synthesis is associated with an increase in polyamine catabolism via the induction of spermidine/spermine acetyltransferase and polyamine oxidase (Cohen 1998). Capell *et al.* (2004) postulate that the polyamine pathway in plants is also regulated at the end-product level. This may be why changes in polyamine contents were not found in the mutants. Capell *et al.* (2004) also ponder on the possibility that if the putrescine pool is built over a certain level, putrescine will be rapidly turned over into the higher polyamines, spermidine and spermine. When overexpression of ADC was increased from 8-fold to 50- fold, spermine accumulated. Basically, if the enzymes in the polyamine pathway are active to a high enough level, the regulation at polyamine end-product level can be overcome (Thu-Hang *et al.* 2002). Perhaps this is true for other aspects of the polyamine pathway. For example, if the input of SAM into the pathway is higher, as with the *mto* mutants, then spermine and spermidine will increase. It has been shown that stress changes polyamine production, perhaps through a change in enzyme activity or threshold levels. Therefore, although there is no significant change in polyamine level under normal conditions, this does not mean that the changes in the polyamine metabolic pathway will not be observed in a different environment. As some of the enzymes for polyamine synthesis are known to be induced by stress, in the next chapter plants will be stressed



to monitor if this will induce a change in polyamine accumulation between wild type and mutant plants.

**Chapter Three: The effects of salt stress on polyamine  
accumulation and on the physiology of the *mto* and *spe*  
mutants**

### 3.1 Aims

There was no difference in the polyamine content of the *spe* and *mto* mutants compared to their respective wild types under normal conditions. Aim of this chapter was to determine the effect of salt stress on the physiology and the polyamine content of the mutants in order to analyse the role of polyamines in salt tolerance. Experiments examined polyamine content under normal (optimal) conditions and under salt stress. Measurements of chlorophyll,  $F_v/F_m$ , polyamine content, fresh and dry weight were taken.

### 3.2 Introduction

#### 3.2.1 Effect of salt stress on polyamine synthesis

Polyamines have been reported to increase or decrease under salt stress, depending on the time of evaluation, species studied and intensity of the stress. Table 4 indicates the current inconsistency of results that fuel the debate about whether polyamines have a role in salt tolerance or not. However, polyamine research may not have contradicted itself, as it seems that polyamine levels are sensitive to growth conditions and even a slight variation may induce different results (Zapata *et al.* 2003). This seems a plausible suggestion considering the many different pathways that polyamines influence.

Although the effects of polyamines on salt tolerance are unclear and so is whether individual polyamines increase or decrease, salt stress has been shown to induce the expression of genes in the polyamine pathway including genes for SAMDC, SPMS, ADC and ODC (Urano *et al.* 2003, Li *et al.* 2003). Interestingly in *Arabidopsis*, *ADC2* has been shown to be induced during salt stress but not *ADC1* (Urano *et al.* 2003). It would be useful to establish a cause-and-effect relationship between enzymes in the polyamine pathway and salt stress.

With the variation in polyamine studies it is difficult to deduce whether polyamines have a role in tolerating salt stress. However, it is easy to imagine how polyamines could have an effect on salt tolerance. For instance, it has been postulated that salt tolerance is dependent on the maintenance of cell membrane integrity, a factor that polyamines are also linked to. The effects of polyamines may not only be cellular. As polyamine levels can change plant physiology and development, this may also change salt tolerance. For example, polyamines have been linked to stomatal movement (Liu *et al.* 2000) which controls transpiration. As cell dehydration is also a consequence of salt stress, control of stomatal movements by polyamines may be important in salt tolerance. Moreover, if polyamines speed up flowering, perhaps this aids plants avoid stress.

It is important to note that media used in these experiments were supplemented with 1% sucrose in addition to salt. Sucrose was added to help plants germinate on the media and thus reach a more homogenous state. Sucrose will affect gene expression. For example in broccoli, increasing sucrose uptake can cause changes in the expression of the ethylene synthetic genes (Nishikawa *et al.* 2005). Furthermore, Ciereszko *et al.* (2004) showed that sucrose can affect gene expression in wild type *Arabidopsis*. They showed that *Ugp* (the gene that encodes UDP-glucose pyrophosphorylase) was upregulated in sucrose fed plants. Moreover, sucrose has been shown to induce flowering at 80 mM (30g/l) and inhibit flowering at concentrations above 110 mM (40g/l) in *Arabidopsis*. An increase from 1% to 1.5% sucrose in the medium correlates with an increase in chlorophyll content, although a comparison without sucrose was not made (Ohto *et al.* 2001). This should not affect the comparison between wild type and mutant plants, although tolerance on salt with sucrose cannot be related directly to tolerance on salt alone.

### **3.3 Materials and Methods**

#### **3.3.1 Plant material**

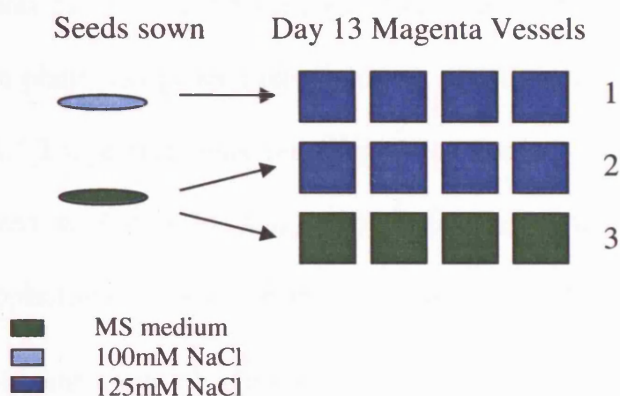
Mutants used in this experiment were outlined in the last chapter

#### **3.3.2 Growth conditions**

All growth vessels were bought pre-sterilised or autoclaved to avoid contamination by other organisms. Media used were autoclaved before pouring under sterile conditions.

Seeds were surface-sterilised by submersion in thin bleach (Tesco) for two minutes. After centrifuging briefly, they were washed three times in sterile distilled water. Seeds were dispersed on 1% agar plates containing half-strength Murashige–Skoog (MS) salts (M5524; Sigma-Aldrich, Irvine, UK) with or without salt (100mM NaCl, 1% (w/v) sucrose) in sterile conditions. Plates were sealed using Micropore surgical tape. After two days in the dark cold room (4°C), seeds were grown in a growth chamber at a 12-h light:12-h dark regime and 50% relative humidity at 22°C in the day and 18°C at night. Light intensity was approximately  $100 \mu\text{mol m}^{-2} \text{s}^{-1}$ . Plates were placed vertically. After 13 days, plants were transferred into magenta vessels containing 1% agar with either salt-containing (125 mM NaCl, 1% (w/v) sucrose) or salt-free MS medium. For each treatment sixteen plants of each genotype were transferred, four plants per magenta vessel (

Figure 12).



**Figure 12.** Method for testing salt tolerance. 3 = normal conditions (Normal), 2 = transfer conditions (Transfer), 1 = salt stress conditions (Salt).

When salt-treated plants began to bleach, plants were analysed for fresh and dry weight,  $F_v/F_m$ , chlorophyll and polyamine content. All readings were taken at midday to minimise variation in polyamine content.

Severe salt stress conditions were simulated by germinating seeds on plates with 125 mM NaCl and transferring them, after 13 days of growth, into magenta vessels containing medium with 150 mM NaCl.

### 3.3.3 Fresh weight and dry weight

Fresh weight was determined directly after plants were removed from magenta vessels. To determine dry weight, plants were dropped immediately into liquid nitrogen and freeze-dried before weighing.

### 3.3.4 Chlorophyll determination

The concentration of chlorophylls *a* and *b* were determined by measuring the absorbance of light by leaf extracts using a spectrophotometer. In 80% acetone, the

absorption maxima are: 663 nm for chlorophyll *a* and 646 nm for chlorophyll *b*. Each plant was placed in a 1.5 ml eppendorf tube to which 1 ml of 80% acetone was added and the plant was ground into solution. 100% acetone was added to adjust the level to 1.5 ml. The extract was centrifuged for 2 min. Absorbance of the leaf extract was measured at 646 nm ( $A_{646}$ ) and at 663 nm ( $A_{663}$ ) using an Ultrospec 3100 pro spectrophotometer (Amersham Biosciences, Little Chalfont, UK).

The concentration of chlorophyll *a* ( $Ca$ ) and *b* ( $Cb$ ) can be calculated using the following equations:

$$Ca = 12.21 * (A_{663} - \text{blank}_{663}) - 2.81 * (A_{646} - \text{blank}_{646}) \text{ in } \mu\text{g ml}^{-1}$$

$$Cb = 20.13 * (A_{646} - \text{blank}_{646}) - 5.03 * (A_{663} - \text{blank}_{663}) \text{ in } \mu\text{g ml}^{-1}$$

$$C_{\text{total}} = 7.18 * (A_{646} - \text{blank}_{646}) + 17.32 * (A_{663} - \text{blank}_{663}) \text{ in } \mu\text{g ml}^{-1}$$

### 3.3.5 Polyamine analysis

Polyamines were determined using the method outlined in chapter two.

### 3.3.6 Maximum photosynthetic efficiency ( $F_v/F_m$ )

Plants were dark adapted for 20 min. Then, still in darkness, they were placed under a kinetic imaging fluorometer (FluorCam, P.S. Instruments, Brno, Czech Republic). This instrument uses high-frequency modulated light to accurately define  $F_0$  and  $F_m$  (Nedbal *et al.* 2000).

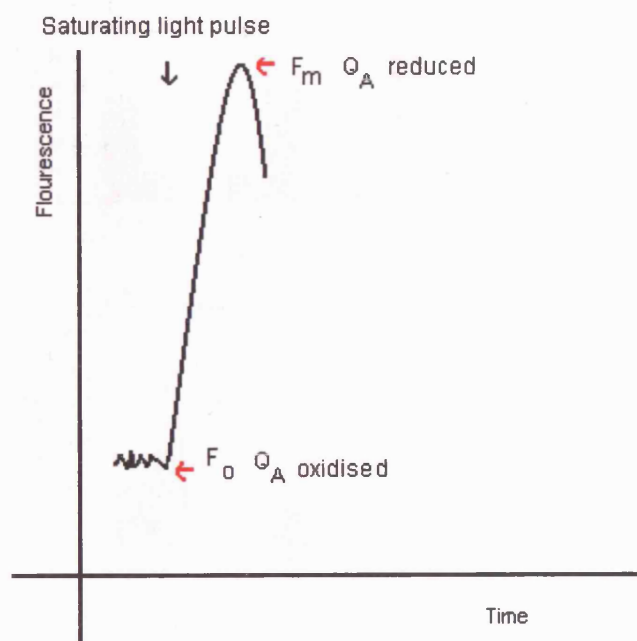
The equation below can be used to determine  $F_v/F_m$ .

$$F_v/F_m = (F_m - F_0) / F_m$$

As the technique is non-invasive, the plants were monitored at day 13 (on plates) and again after bleaching begun (on magenta vessels).  $F_v/F_m$  is generally related to plant

health as it reflects the efficiency of photosystem II and the ability of plants to move energy through the system, a value of 0.8 indicates a unstressed healthy plant.

As plants absorb light, electrons are excited. This energy can be channelled into photosynthetic PS electron transport, heat or fluorescence.  $F_0$  is determined by a series of low intensity measuring flashes (Figure 13). When  $Q_A$  is oxidised, the plant can use the energy from light and move it along photosystem II, this means the excitation lost to fluorescence is low.  $F_m$  was determined during a saturating pulse (ca.  $1800 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ , duration 1-2 s) and represents the value when  $Q_A$  is reduced and the fluorescence is at a maximum, as energy cannot flow through the system.  $F_v$  is the difference between these values.



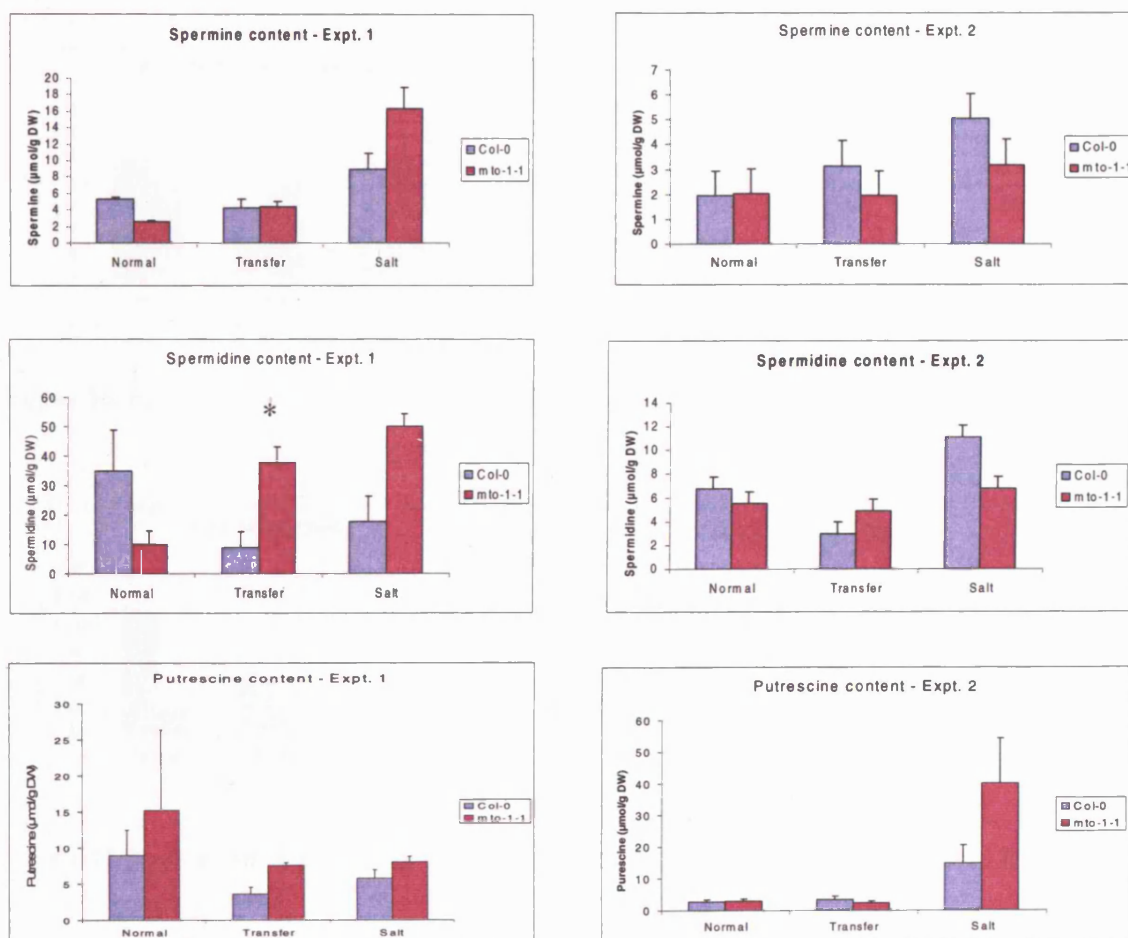
**Figure 13** Determination of  $F_v/F_m$



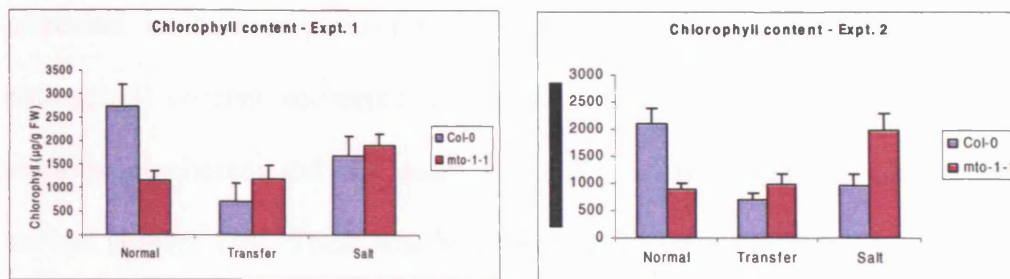
### 3.4 Results

The experiments detailed below were repeated several times. The results below summarize the data from two experiments, which show the variation in results.

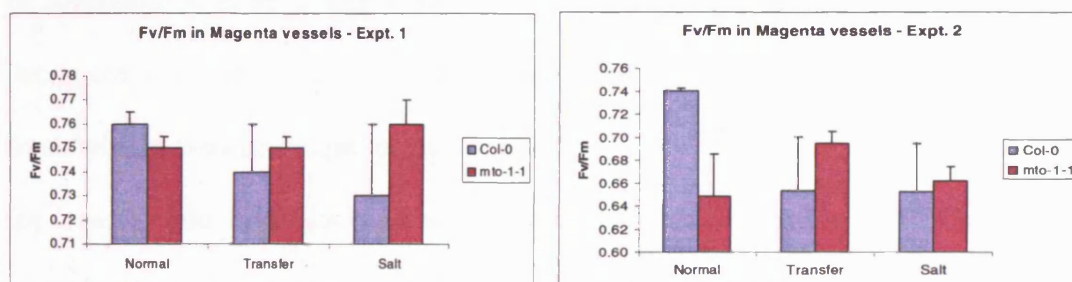
#### 3.4.1 Col-0, *mtol-1*



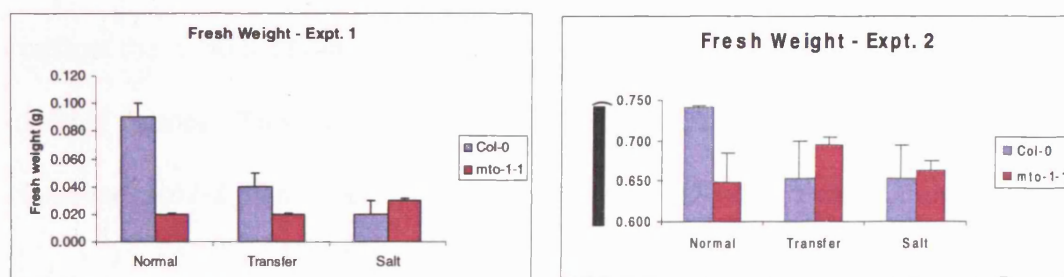
**Figure 14.** Polyamine content in Col-0 and *mtol-1* plants grown on half strength MS medium (Normal), grown under normal conditions then transferred to medium containing 100mM NaCl (Transfer) and grown on medium with 100mM NaCl and transferred to medium with 125mM NaCl (Salt). \* denotes significant difference (t test,  $P < 0.05$ , four replicates) between mutant and wild type. Error bars show standard error values.



**Figure 15.** Chlorophyll content in *mto1-1* and Col-0 grown under the conditions outlined in Figure 14.



**Figure 16.**  $F_v/F_m$  in *mto1-1* and Col-0 grown under the conditions outlined in Figure 14.

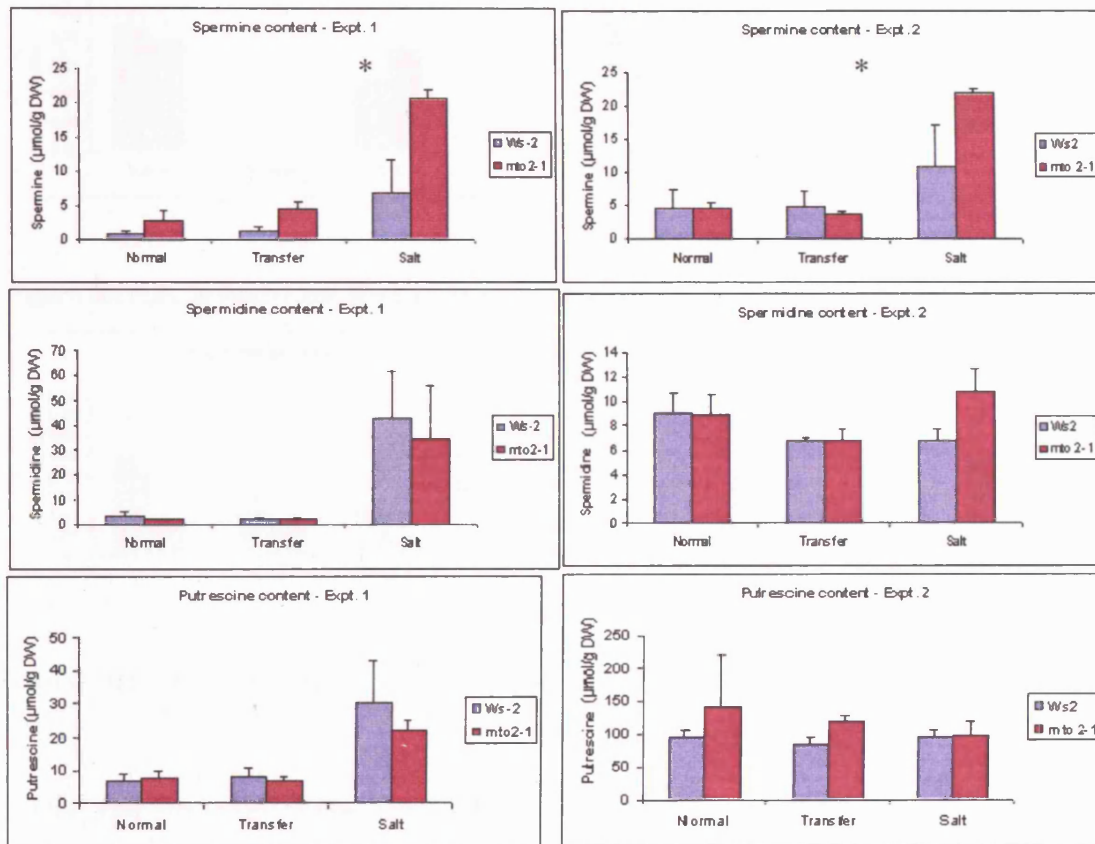


**Figure 17.** Fresh weight in *mto1-1* and Col-0 grown under the conditions outlined in Figure 14.

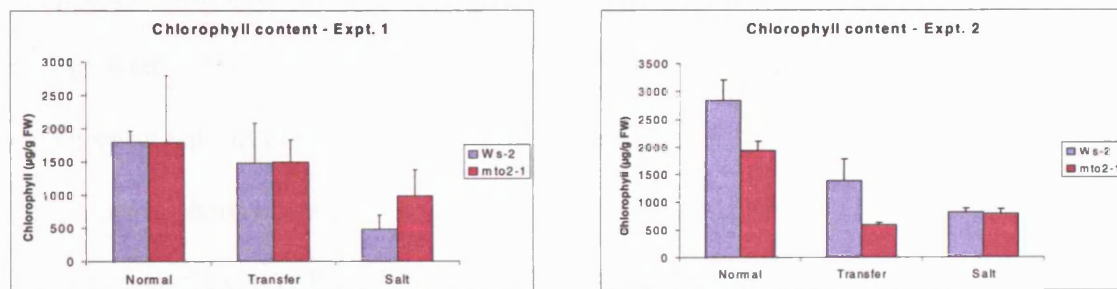
In the first experiment (Figure 14) it seems that there was an induction of the polyamines spermidine in *mto1-1* under salt stress conditions. Similar results are not seen in experiment two. The increase in putrescine in experiment two was seen both in the wild type and the mutant and may be a physiological response. As reflected in these examples, even by keeping the environment controlled, consistent results were not obtained, although it is apparent that the total polyamine content in *mto1-1* had

increased in most experiments more than in Col-0. In both experiments, the chlorophyll content decreased during salt stress in Col-0 (Figure 15). In *mtol-1*, chlorophyll content did not decrease.  $F_v/F_m$  values were determined before plant harvest (Figure 16). There was no consistent difference in  $F_v/F_m$  between mutant and wild type or between treatments. This is perhaps because salt stress was not severe enough to stress the wild type. Interestingly, Col-0, when compared to other ecotypes of *Arabidopsis*, such as Ler-0, seems more tolerant. The fresh weight of Col-0 plants decreased whereas it stayed the same in *mtol-1* plants (Figure 17). The difference in fresh weight between experiment one and two is due to variations in the plants chosen for transfer into magenta vessels. The decline in the average values of spermine and spermidine over time and the increase in putrescine are possibly environmental and due to the life cycle of the plant. It has been shown that polyamine contents change before flowering (Martin-Tanguy 1997). However, repeat experiments should be carried out to confirm these fluctuations in polyamines are due to the plant's life cycle and not to external factors. This trend was seen in four experiments out of five (see Appendix). However, *mtol-1* plants were smaller than Col-0 plants under normal conditions.

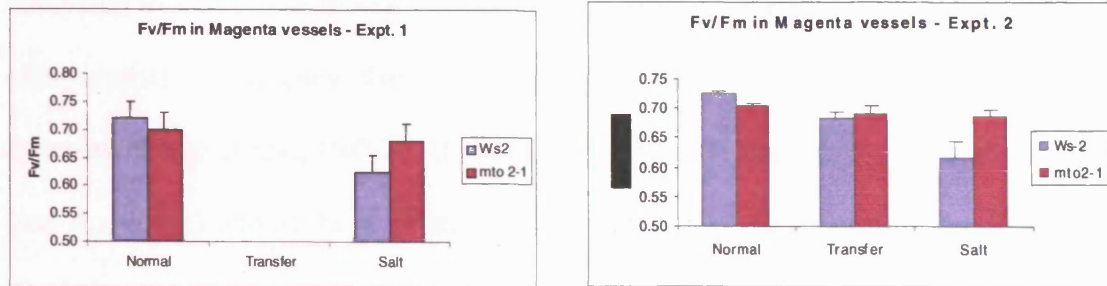
### 3.4.2 Ws-2, *mto2-1*



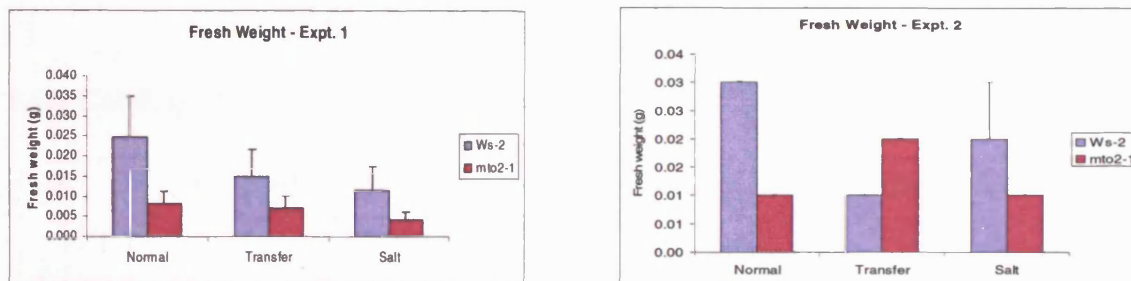
**Figure 18.** Polyamine content in Ws-2 and *mto2-1* plants grown on half strength MS medium (Normal), grown on normal conditions then transferred to medium containing 100mM NaCl (Transfer) and grown on medium with 100mM NaCl and transferred to medium with 125mM NaCl (Salt). \* denotes significant difference (t test,  $P < 0.05$ , four replicates) between mutant and wild type. Error bars show standard error values.



**Figure 19.** Chlorophyll content in *mto2-1* and Ws-2 grown under the conditions outlined in Figure 18.



**Figure 20.**  $F_v/F_m$  in *mto2-1* and Ws-2 grown under the conditions outlined in Figure 14.



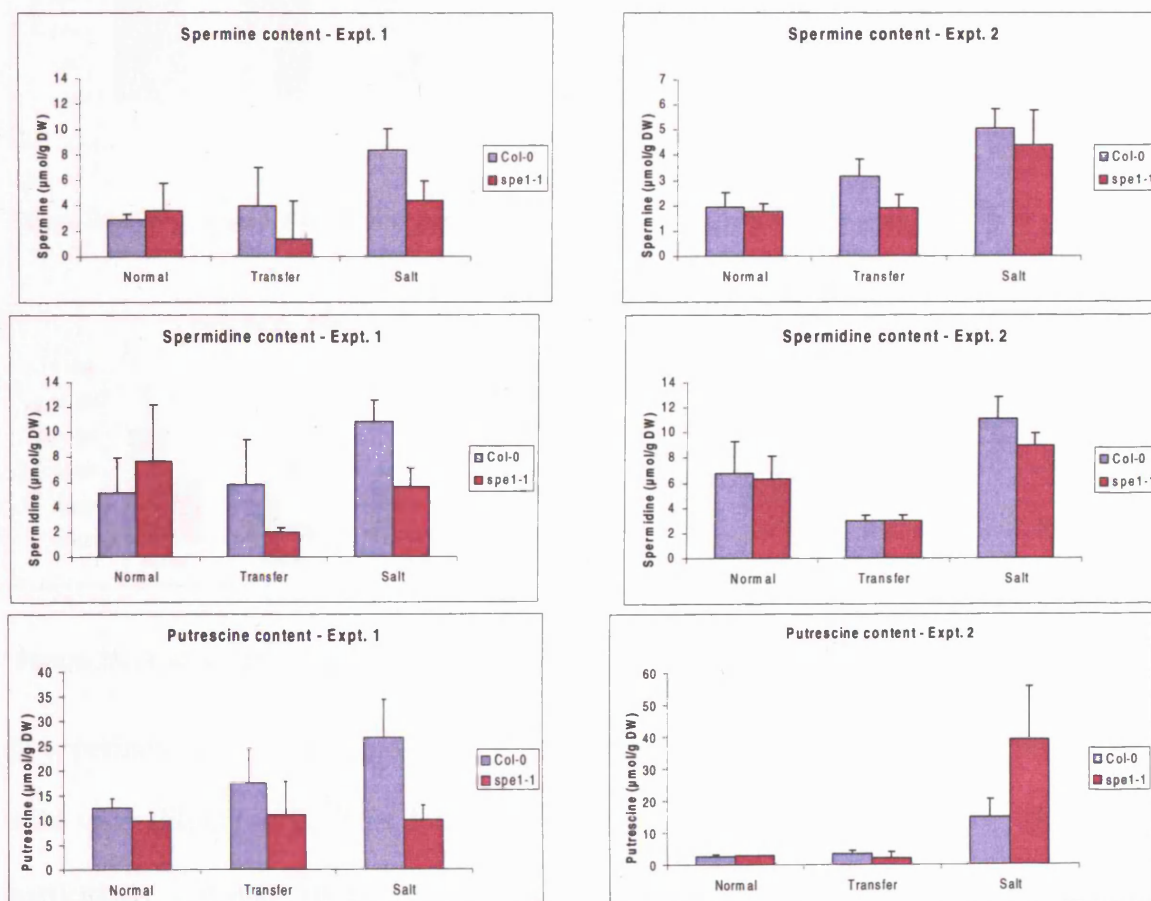
**Figure 21.** Fresh weight in *mto2-1* and Ws-2 grown under the conditions outlined in Figure 14.

Under salt stress (pre-treatment with 100 mM NaCl then transfer onto 125 mM NaCl, Figure 18) both *mto2-1* and Ws-2 plants accumulated polyamines. This accumulation was not seen in plants that were grown on normal growth conditions or those that were transferred from normal medium to medium containing 125 mM NaCl. This could be as the plants have not had time to respond to the saline environment or perhaps detection of salt on germination changes the regulation of metabolic pathways. In both experiments, there was a higher spermine content under saline conditions in *mto2-1* than in Ws-2 (t-test, expt 1.  $P=0.039$ , expt 2.  $P=0.047$ ). In experiment one, spermidine and putrescine were induced in both mutant and wild type, this induction was not seen in experiment two. Putrescine values in experiment two were much higher than in experiment one, despite similar spermine values. This could be due to a different time of harvesting or due to environmental variation caused by slight differences in the media (e.g. pH). Chlorophyll content was not significantly different in *mto2-1*

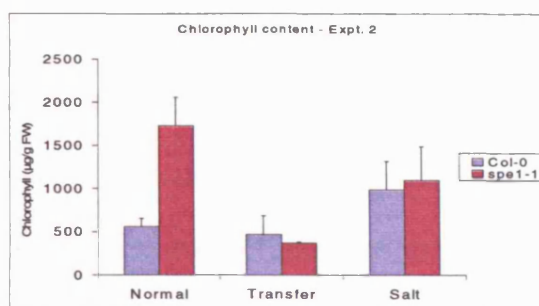
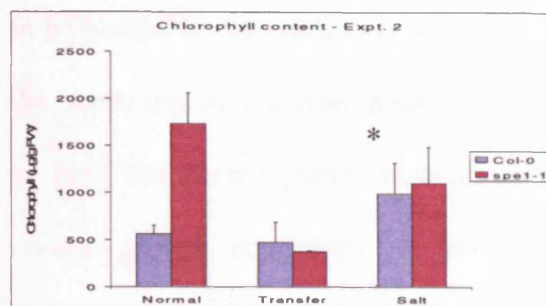


compared to Ws-2 in both experiments (Figure 19). *mto2-1* showed less of a decline in photosynthetic efficiency than wild type plants when placed under salt stress in experiment two (t test,  $P=0.01$ ) (Figure 20). The difference was seen both at day 13 (see Appendix) and at the later stage (Figure 20). As *mto2-1* grew more slowly than Ws-2, its fresh weight in the normal treatment was slightly lower. However, under salt stress the fresh weight of *mto2-1* plants did not decline as in Ws-2 (Figure 21).

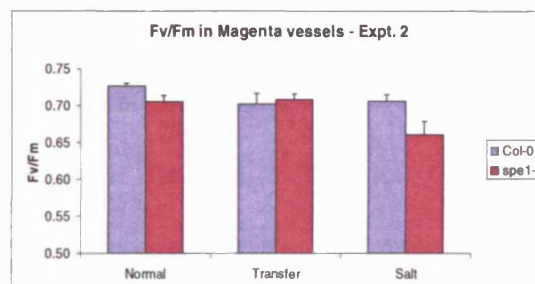
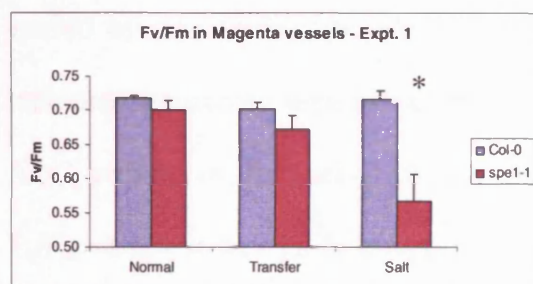
### 3.4.3 Col-0, *spe1-1*



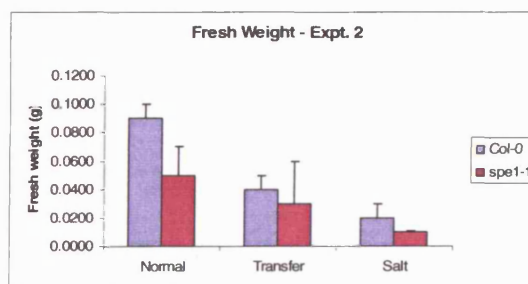
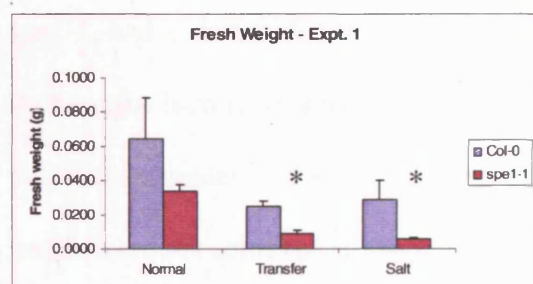
**Figure 22.** Polyamine content in Col-0 and *spe1-1* plants grown on half strength MS medium (Normal), grown on normal conditions then transferred to medium containing 100mM NaCl (Transfer) and grown on medium with 100mM NaCl and transferred to medium with 125mM NaCl (Salt). Error bars show standard error values.



**Figure 23.** Chlorophyll content in *spe1-1* and Col-0 grown under the conditions outlined in Figure 22.



**Figure 24.**  $F_v/F_m$  in *spe1-1* and Col-0 grown under the conditions outlined in Figure 22.



**Figure 25.** Fresh weight in *spe1-1* and Col-0 grown under the conditions outlined in Figure 22.

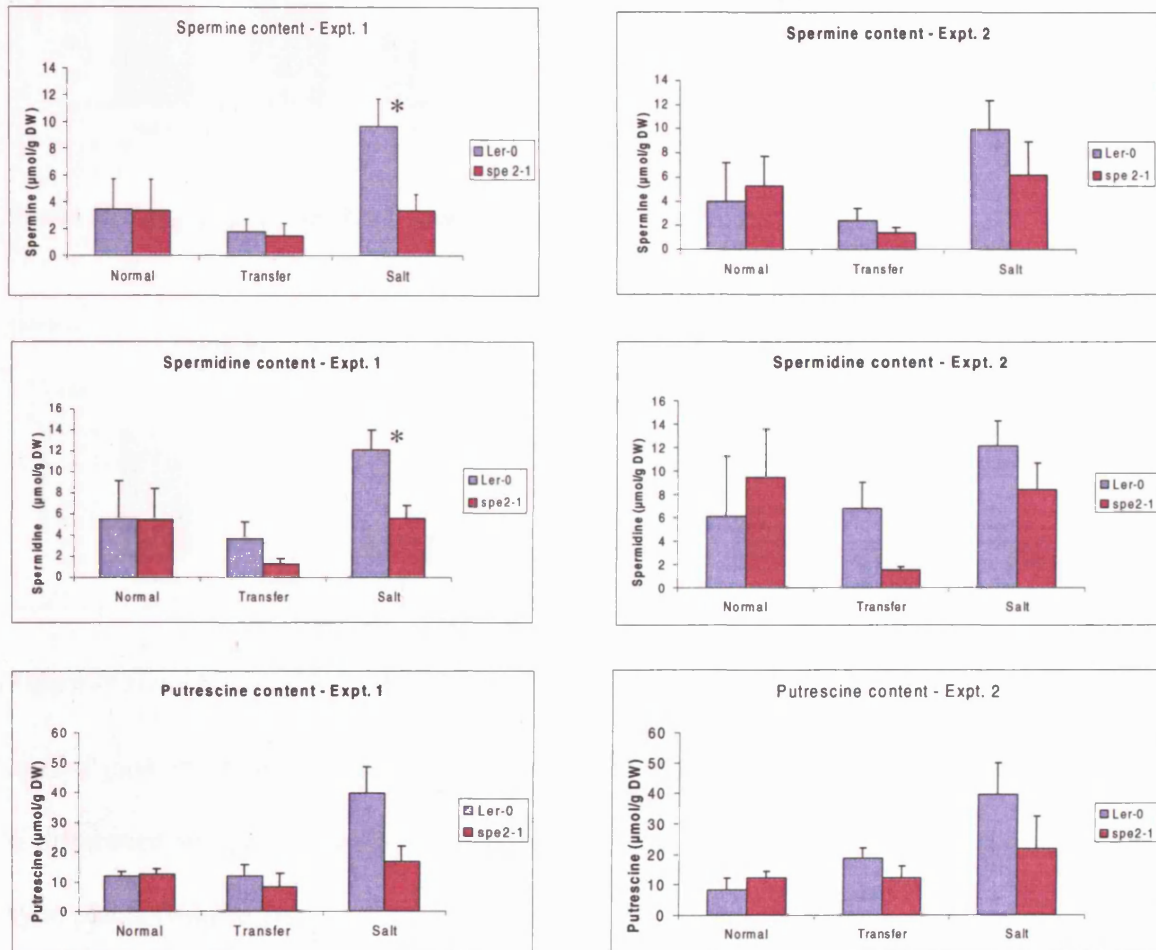
In experiment one, there was an overall induction of polyamines due to salt stress in the wild type (Figure 22). This induction was not seen with *spe1-1*. This effect was particularly apparent in putrescine. This was difficult to reproduce, as seen in experiment two. Here, there was no significant difference between spermine and spermidine content in stressed and normal conditions in either mutant or wild type. Putrescine increased in both the mutant and the wild type in experiment two far more than in experiment one.

In both experiments, although the salt treatment induced a change in polyamine content, the plants transferred from normal to salt-containing medium showed no such increase. In fact, transferred plants showed similar polyamine levels to plants grown under normal growth conditions. In experiment one, the chlorophyll content in *spe1-1* decreased more than in the wild type (Figure 23). In experiment two, there seemed to be a low value for Col-0 chlorophyll under normal conditions, this could be due to damage caused by transferring the plants from plates to magenta vessels (Figure 23). The increased sensitivity seen in experiment one, which could be caused by the decreased ADC activity in the *spe1-1* mutant, is also reflected in the  $F_v/F_m$ . With *spe1-1*, the  $F_v/F_m$  values at day 13 (see Appendix) showed no difference between the mutant and the wild type, but later in development *spe1-1* had a lower  $F_v/F_m$  than its wild type (Figure 24).

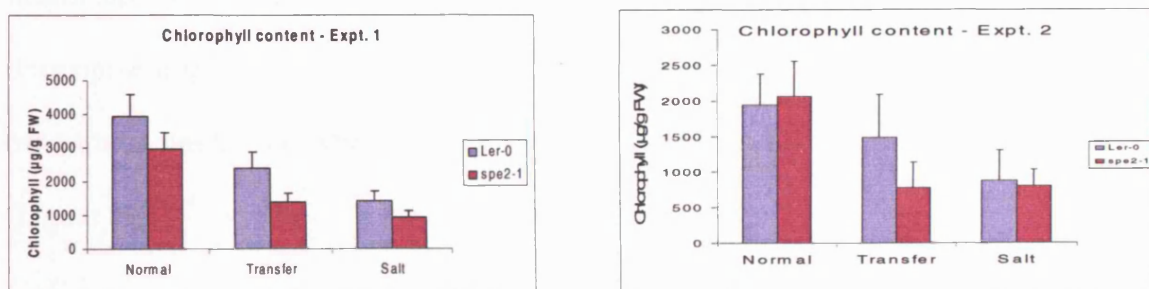
*spe1-1* had a lower fresh weight when grown under saline conditions (Figure 25). Perhaps the increased sensitivity of *spe1-1* to salt stress was due to its inability to induce polyamines under salt stress (Figure 22, experiment one). Once more, as consistent trends were not achieved in the polyamine data, this is uncertain.



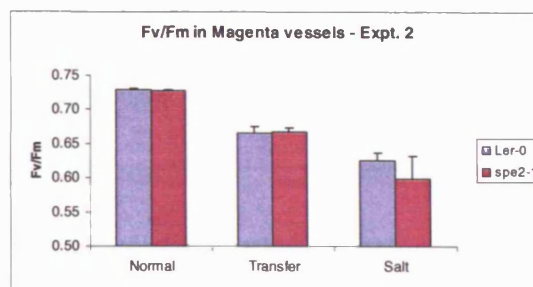
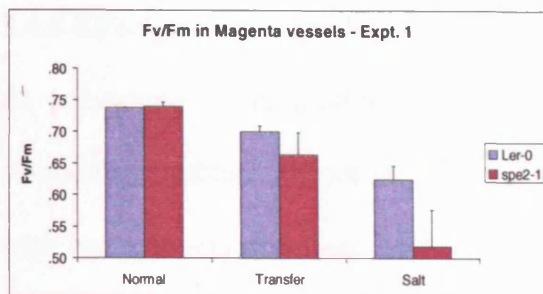
### 3.4.4 Ler-0, *spe2-1*



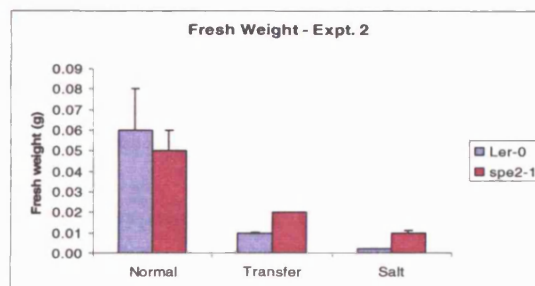
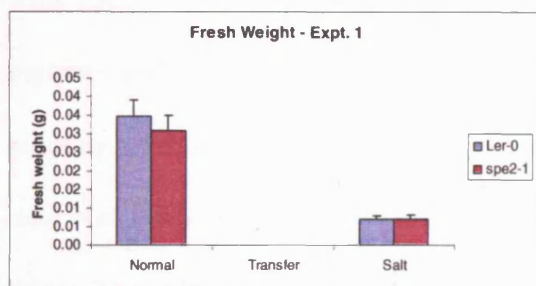
**Figure 26** Polyamine content in Ler-0 and *spe2-1* plants grown on half strength MS medium (Normal), grown on normal conditions then transferred to medium containing 100mM NaCl (Transfer) and grown on medium with 100mM NaCl and transferred to medium with 125mM NaCl (Salt). \* denotes significant difference (t test,  $P < 0.05$ , four replicates) between mutant and wild type. Error bars show standard error values.



**Figure 27** Chlorophyll content in *spe2-1* and Ler-0 under conditions outlined in Figure 26.



**Figure 28**  $F_v/F_m$  in *spe2-1* and Ler-0 under conditions outlined in Figure 26.



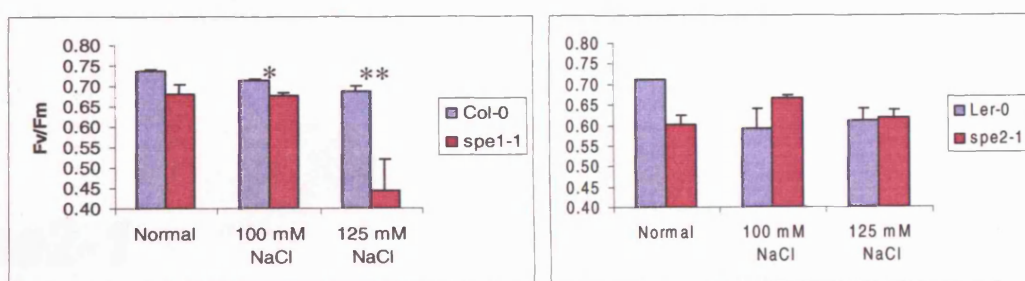
**Figure 29** Fresh weight in *spe2-1* and Ler-0 grown under conditions outlined in Figure 26.

*spe2-1* gave the most consistent results of all the mutants. In experiment one, there was a difference in spermidine and spermine values between salt-treated mutant and wild type plants (Figure 26).

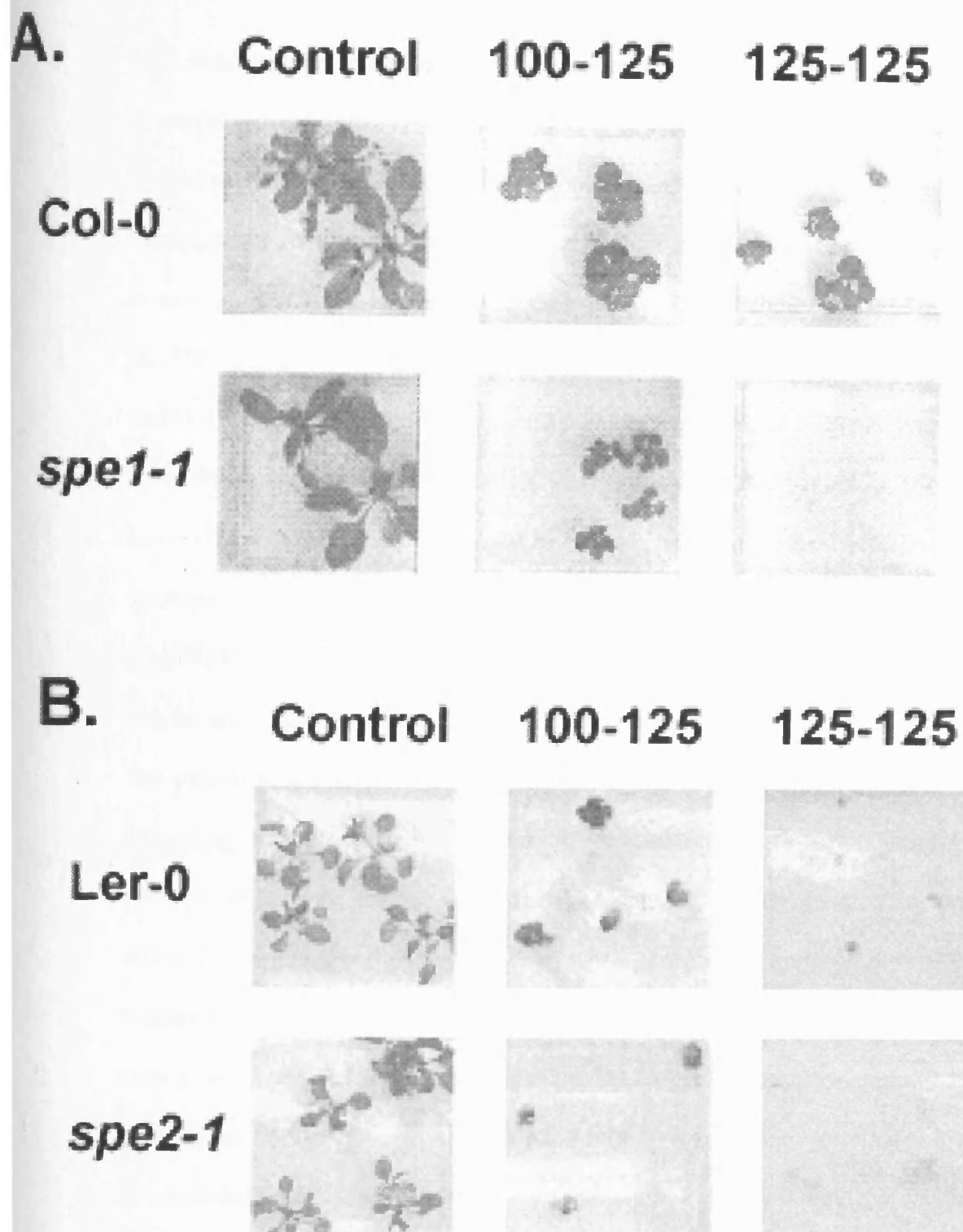
The chlorophyll content decreased significantly under saline conditions in both Ler-0 and *spe2-1* reflecting the sensitivity of this genetic background (Figure 27). The chlorophyll content stayed the same between mutant and wild type in the different treatments.  $F_v/F_m$  values showed no difference at day 13 (data not shown) but later in development *spe2-1* had lower values than Ler-0 on salt containing medium (Figure 28, experiment one). Salt stress caused the fresh weight to decline in *spe2-1* and Ler-0 (Figure 29).

### 3.4.5 Effect of severe salt stress in the *spe* mutants

As polyamine accumulation was only found in plants that were germinated on salt-containing medium but not in plants transferred from normal conditions onto medium with NaCl, it was examined how a more severe salt stress affects plant growth. Severe salt stress was imposed by germinating seeds on plates with 125 mM NaCl and transferring them, after 13 days of growth, into magenta vessels containing medium with 150 mM NaCl. Figure 31 highlights the difference in tolerance between the *spe* mutants and their respective wild types. When germinated on 100 mM NaCl, the *spe* mutants were slightly smaller than the wild type. However, when germinated on 125 mM NaCl the *spe* mutants were much smaller and bleached faster than the wild type lines. The difference between Col-0 and *spe1-1* was more dramatic than the difference between Ler-0 and *spe2-1*, due to the difference in salt sensitivity of these background lines. This is reflected in the  $F_v/F_m$  at day 13 of these lines (Figure 30). With *spe1-1*, plants had a lower  $F_v/F_m$  values than wild type when grown on 100 mM NaCl and when grown at 125 mM NaCl, this difference increased. Conversely, with *spe2-1*, there was no difference between the mutant and the wild type under salt stress.



**Figure 30.**  $F_v/F_m$  at day 13 of Col-0, Ler-0, *spe2-1* and *spe1-1* grown on normal MS medium, and medium containing 100 mM and 125 mM NaCl. \* denotes significant difference (t test,  $P < 0.05$ , twenty replicates) between mutant and wild type. Error bars show standard error values.



**Figure 31.** Effect of severe salt stress in the *spe* mutants.

## 3.5 Discussion

### 3.5.1 Polyamine accumulation in *Arabidopsis* under salt stress

In normal growth conditions, there was no difference in polyamine content between mutant and wild type plants (Chapter Two). Under salt stress, polyamines accumulated in mutants and wild type lines. This could be as polyamines act as an osmoprotectant or as a cause of stress-induced injury (Jouve *et al.* 2004). Furthermore, Brüggermann *et al.* (1998) postulated that common polyamines can mediate a salt stress-induced decrease of ion flux across the vacuolar membrane by blocking fast-activating vacuolar channels and decreasing the cation permeability of the tonoplast. Interestingly, spermine and spermidine were shown to be at least 100 times more effective at blocking channels than putrescine. It is often postulated that spermine and spermidine are more effective than putrescine as protectants (Zapata *et al.* 2003), yet results presented in this chapter do not indicate differences in the response of individual polyamines to stress.

The induction of polyamines was seen when the plants were sown directly onto salt-containing medium, but not when they were transferred onto salt-containing medium after germination on normal medium. What happens during germination on salt that allows polyamine accumulation to occur, when germination under in normal conditions followed by transfer to salt does not result in polyamine accumulation?

Induction of polyamine accumulation may be triggered on germination, or perhaps polyamines are induced above a certain ion threshold and plants germinated on salt have accumulated more ions. Alternatively, acclimation may be required for polyamine accumulation. It could be that by germinating a plant under saline conditions, plants will begin to form precursors of polyamine synthesis at a higher rate than plants germinated under normal conditions (especially if one polyamine pool needs to reach a

certain level before the others can accumulate). Germination on salt may give plants a longer time to increase the levels of proteins (e.g. enzymes) involved in polyamine synthesis. It was shown in rice that SAMDC transcript levels only reached the maximum level after six days of stress induction (Capell *et al.* 2004). It could be that upon germination, plants sense their surroundings and adjust their metabolism to be stress sensitive and that plants that have not made this adjustment take longer to react to stress.

The effects of germination under saline conditions on *Arabidopsis* growth were described by West *et al.* (2004), who showed that cell production and cell cycle progression were inhibited and that meristem size was reduced. Cell activities may return to normal but the meristem reduction is permanent. This indicates that by germinating seeds on salt-supplemented medium, overall plant physiology changes. It is well known that polyamines affect cell division, thus an increase in polyamine content may be a response to the reduction of meristem size. It would be interesting to see whether germination on salt, followed by transfer to normal conditions changes polyamine metabolism. Furthermore, experiments could be done to show what salt concentrations are required at germination to induce polyamines or if other osmotically active compounds can also induce polyamine accumulation. Early polyamine response would also be interesting to look at. Time-course experiments could be done to see how fast each polyamine accumulates after germination on salt and whether one polyamine needs to accumulate before another.

It is not only the germination environment that determines the plant's adaptability to stress. The tolerance of each wild type line varies. This is particularly apparent in the severe stress experiments with the *spe* mutants where the Col-0 background was more

tolerant than the Ler-0. The natural variation in salt tolerance in *Arabidopsis* could be utilized for determining the genetic background of salt tolerance.

### **3.5.2 Role of methionine overproduction in polyamine accumulation and salt tolerance**

There was no increase in polyamine content in the *mto* mutants under normal conditions compared to their wild types. It has been suggested that SAMDC is a rate determining enzyme which would explain why in normal conditions, an increase in methionine does not increase the polyamine content (Waie *et al.* 2003).

#### ***mto1-1***

Overall, *mto1-1* did not show a significant difference in salt tolerance compared to Col-0. The experiments do not show a reliable trend indicating that *mto1-1* has a higher polyamine content compared to Col-0, although a significant increase in spermidine content of the mutant was seen under stress conditions in experiment one. Therefore, due to high variation, these experiments cannot determine whether polyamines and salt tolerance are related or not. This confirms that polyamine accumulation is highly dependent on the condition of the plants, the level of stress induced and the time of sampling.

#### ***mto2-1***

Under salt stress, *mto2-1* had an increased spermine content with respect to Ws-2. It also showed less of a decline in photosynthetic efficiency at days 13 and 20 in experiment two. Spermine has been shown to prevent chlorophyll loss (Chattopadhyay *et al.* 2002), so the elevated content of spermine in the salt-stressed plant may protect



the thylakoid membranes. Methionine is a precursor of SAM, which donates the aminopropyl group required for formation of spermine and spermidine, yet only spermine accumulated in both experiments. This could be as spermine is the last polyamine in the pathway or perhaps if methionine supply is limited, spermine accumulation is preferred. Alternatively, maybe the levels of spermidine are more stringently controlled.

### **3.5.3 Role of arginine decarboxylase in polyamine accumulation and salt tolerance**

Salt tolerance was reduced in the *spe* mutants compared to their respective wild types. This is reflected in Figure 31 when plants were subjected to severe salt stress. Decreased salt tolerance may be due to the decrease in ADC activity in these mutants, although it cannot be excluded that alterations in root growth (Watson *et al.* 1998) could have affected the uptake of salt and thus salt tolerance. Furthermore, the *spe* mutants should be sequenced, this would confirm that *spe2-1* was an *ADC2* mutant and would test whether *spe1-1* was an *ADC1* mutant or not

#### ***spe1-1***

In general, *spe1-1* had a lower fresh weight,  $F_v/F_m$  and chlorophyll content than Col-0 under saline conditions. Severe salt stress caused bleaching to occur more readily in *spe1-1* than in Col-0, reflecting that this mutant was more sensitive to salt stress than its wild type. This shows that the gene affected by this mutation is important in aiding the tolerance of plants to salt stress. Whether tolerance was related to polyamine content is debatable, as individual polyamine responses vary between experiments. It could be that this mutation affects another compound(s) related to the polyamine pathway (such as conjugated polyamine levels), which were not examined in this thesis. Certainly if



the mutation is related to transcriptional and/or post-transcriptional regulation it could affect many genes.

As *spe1-1* is not mapped, it would be interesting to see if the phenotype observed under severe salt stress could be used to locate the mutation. To exclude a mutation in the *ADC1* gene, this gene could be sequenced and compared against the *Arabidopsis* database. If it is not a mutation in the *ADC1* gene then a segregating F<sub>2</sub> population would have to be created first to see if this is possible. This population would have to be analysed for known genetic markers to see if the phenotype is linked to one of these markers.

### ***spe2-1***

*spe2-1* carries a mutation mapping to the *ADC2* gene. Under saline conditions, polyamines were induced in Ler-0 and not in *spe2-1*. Under normal conditions the chlorophyll content,  $F_v/F_m$  and the fresh weight of *Arabidopsis* was not affected by a decline in ADC2 activity. Under saline conditions, no repeatable trends were found in the experiments, this may be as it was difficult to detect a difference as Ler-0 (the background line) was very sensitive to salt. Under severe salt stress, bleaching occurred more readily in *spe2-1* than in Ler-0, demonstrating that this mutant was more sensitive to salt stress than its wild type. As the mutant had a lower polyamine content than the wild type under salt stress and had a higher sensitivity to salt, it is safe to conclude that the ADC2 enzyme is important for salt tolerance. Polyamines are said to protect against chlorophyll loss. Chattopadhyay *et al.* (1997) showed that loss of membrane integrity as well as PSII electron transport was diminished when exogenous polyamines were added. This could explain the effects on  $F_v/F_m$ , which mirrors photosynthetic

efficiency. Perhaps, the decreased polyamine content in *spe2-1* mutant decreases the protection of the LHC and PSII complexes in the thylakoids (Navakoudis *et al.* 2003).

*ADC2* has been induced during osmotic stress (Soyka *et al.* 1999). Furthermore, Urano *et al.* (2003) showed that *ADC2* and *SPMS* mRNA increased under saline condition and that *ADC2* accumulation correlated with putrescine accumulation. Here, with *spe2-1* it was shown that in both experiments putrescine was lower in the mutant than the wild type. In experiment one, there was no induction of spermine and spermidine observed in *spe2-1* as in Ler-0, the difference between mutant and wild type was not as clear in experiment two. The difference in results could be dependent on time of sampling. Tonon *et al.* (2004) showed that polyamines vary under short and long term salt stress and upon the type of stress (osmotic or ionic). Furthermore, to confirm that this mutant is an *ADC2* mutant, the *ADC2* gene could be sequenced.

It is conceivable that spermine and spermidine contents cannot increase in saline conditions if there is no increase in putrescine. It has been shown that an increase in spermine and spermidine is dependent on the putrescine pool reaching a certain threshold level in rice (Capell *et al.* 2004). If this theory is true, then the *spe* mutants, would take longer to increase the putrescine level to above this critical threshold level and would therefore take longer to increase spermine and spermidine contents. Alternatively, if putrescine formation was the rate-determining step in increasing polyamine synthesis, then it may not be surprising that the *mto* mutants did not show a consistent increase in polyamine accumulation. It would be fascinating to investigate if this is true and see how other polyamine synthesis enzymes are involved in salt tolerance, perhaps by doing similar experiments with mutants in *SPMS* and *SPDS*.

## **Chapter Four: The effects of osmotic stress on the *mto* mutants**

## 4.1 Aim

The aim of this chapter was to determine if over-production of methionine affects polyamine accumulation and tolerance of osmotic stress. Although the *mto* mutants did not show a consistently higher accumulation of polyamines under salt stress, they may do under longer periods of stress. Osmotic stress was studied as it is easy to simulate over a long period of time.

## 4.2 Introduction

### 4.2.1 Osmotic Stress

Osmotic stress causes changes in root metabolism sending signals to the shoots, which respond by modulating gene expression. Under mild stress conditions, this leads to the recovery of cell homeostasis. If stress is more severe, the result is a decrease in growth, stomatal conductance, photosynthesis and osmotic potential (Kramer and Boyer 1995, Nepomuceno *et al.* 1998).

Osmotic stress causes cell dehydration. This is commonly caused by a decrease in water potential imbalance between the apoplast and symplast. This can decrease cell turgor, which can have an effect on plant growth. As turgidity decreases many metabolic changes occur (Xiong *et al.* 2002) including a decrease in photosynthesis and carbon assimilation (Nepomuceno *et al.* 1998). Plants can prevent this by osmotic adjustment through the use of cell osmolytes. Osmolytes are thought to adjust the cellular osmotic potential to increase water potential but they have also been linked to membrane integrity (Rhodes and Hanson 1993) and the scavenging of excess reactive oxygen species (Bohnert and Jensen 1996). Osmolytes in plants include glycine betaine, trehalose, proline, ononitol, mannitol, sorbitol and polyamines (Nuccio *et al.* 1999).

Salt and osmotic stress share a number of plant responses as they both cause cellular dehydration. For instance, they both induce accumulation of certain osmolytes, such as proline and water soluble carbohydrates (Kerepesi *et al.* 2000). The difference between osmotic and salt stress is that there is no ionic factor in osmotic stress. Excess salt causes an increase of  $\text{Na}^+$ , which is a cytotoxin and limits the import of  $\text{K}^+$ . Therefore, although some responses to salt and osmotic stress are invariably linked, it cannot be assumed that plants will respond to salt stress in the same fashion as to osmotic stress.

Donaldson *et al.* (2004) raised the possibility that a cGMP-dependent  $\text{Ca}^{2+}$  increase was only seen in salt stress and not in osmotic stress. This leads to the possibility of different signalling pathways being involved in each stress causing plants to respond in different manners. This is certainly the case in rice, where Bahaji *et al.* (2003) looked at the relationship between osmotic and saline stress. They concluded that both stresses cause a similar pattern of endocytosis (consisting of an initial inhibition of up-take and then later activation) but differed in the level of endocytosis needed for tolerance. Salt tolerance required reduced endocytosis, but osmotic tolerance required increased endocytosis.

Furthermore, Ueda *et al.* (2004) showed that there were 53 genes that showed differential expression under osmotic stress, compared to 92 genes under salt stress. 18 of these genes showed a vastly different expression between salt and osmotic stress.

Polyamines have been linked to osmotic stress tolerance. Capell (2004) showed that rice mutants that over-produced polyamines were tolerant to osmotic stress. This tolerance could be due to many factors. For example, polyamines are known to be stabilizing factors of membranes and macromolecules. It has been shown that addition of exogenous polyamines reduces the destruction of the thylakoids and plastids and also prevents the loss of pigments (Navakoudis *et al.* 2003). Furthermore, polyamines have

been linked to stomatal movements. On addition of polyethylene glycol (PEG), *Arabidopsis* reduces water loss by the closure of the stomata; this can help alleviate cell dehydration brought on by osmotic stress (Leymarie *et al.* 1999). As stress disrupts cellular homeostasis, it changes chloroplast functions and can lead to the generation of reactive oxygen species. Polyamines have been linked to free radical scavenging (Besford *et al.* 1993). Considering the above, it would be interesting to see if there is a link between polyamines and osmotic stress tolerance.

PEG is a non-ionic, long chain inert polymer which can be used to simulate osmotic stress. PEG solutions have an increased viscosity, which have been shown to hinder oxygen diffusion to the root (Verslues *et al.* 1998). PEG affects *Arabidopsis* by inducing the elongation of the primary root and the rate of cell production (Van der Weele 2000).

### **4.3 Materials and methods**

#### **4.3.1 Plant Material**

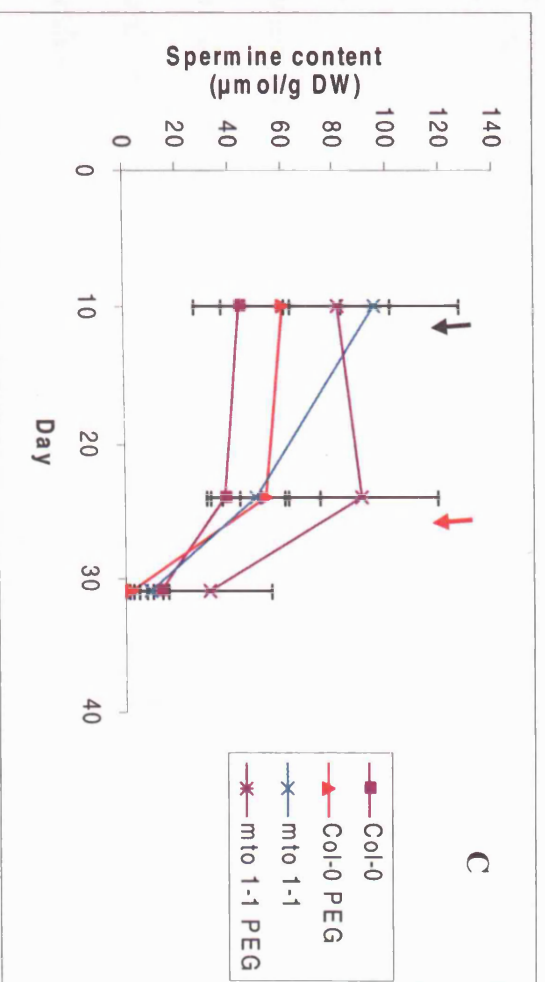
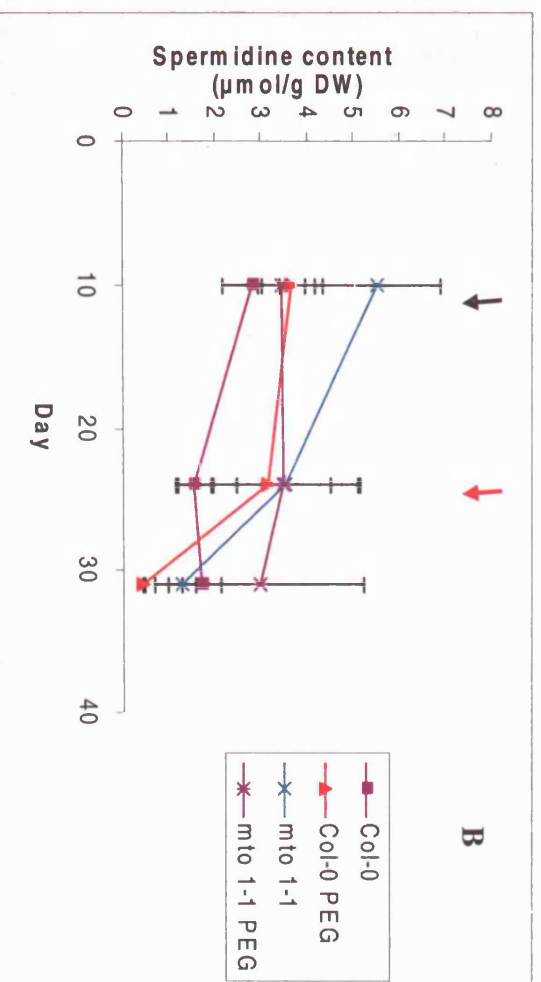
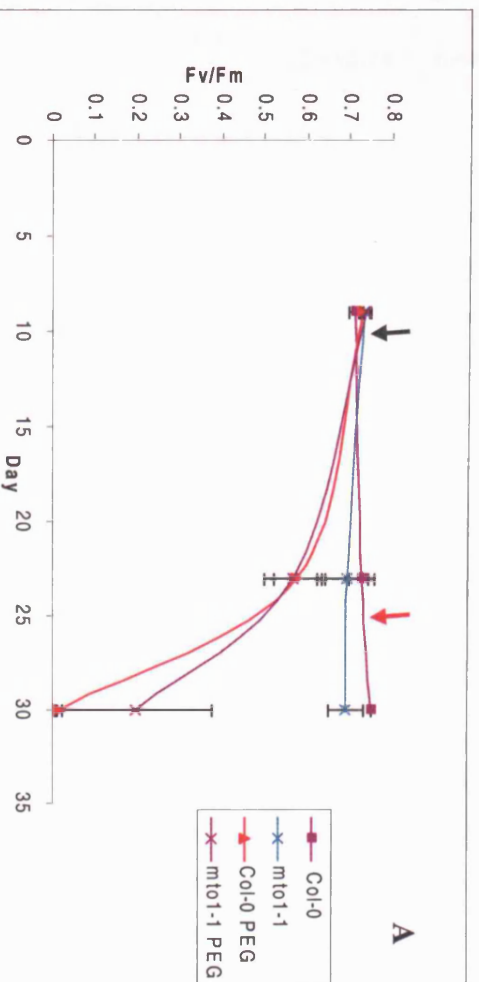
The mutants *mtol-1* and *mtol2-1* were observed, along with their respective wild types Col-0 and Ws-2. Van der Weele (2000) showed that incorporating high molecular weight solutes (such as PEG) into agar was difficult, as larger compounds interfered with the polysaccharide matrix and inhibited gelling. Therefore, in this experiment, agar medium was replaced with vermiculite and plant pots were used. Seeds were sown on soil, left in the cold room for two days and grown in growth chambers under a 12-h light:12-h dark regime and 50% relative humidity at 22°C at day and 18°C at night for two weeks. Light intensity was approximately  $100 \mu\text{mol m}^{-2} \text{s}^{-1}$ . Twelve mutant and twelve wild type plants of similar size were picked and re-potted into vermiculite. These were grown for one week in 40% Long Ashton solution (Hewitt *et al.*, 1966). At week two, half the plants (six from mutant and six from the wild type) were osmotically stressed by watering them with 40% Long Ashton solution containing 10% (w/v) polyethylene glycol MW 10,000 (PEG). After three weeks the concentration of PEG was increased to 12% (w/v).

#### **4.3.2 Polyamine analysis and maximum photosynthetic efficiency ( $F_v/F_m$ )**

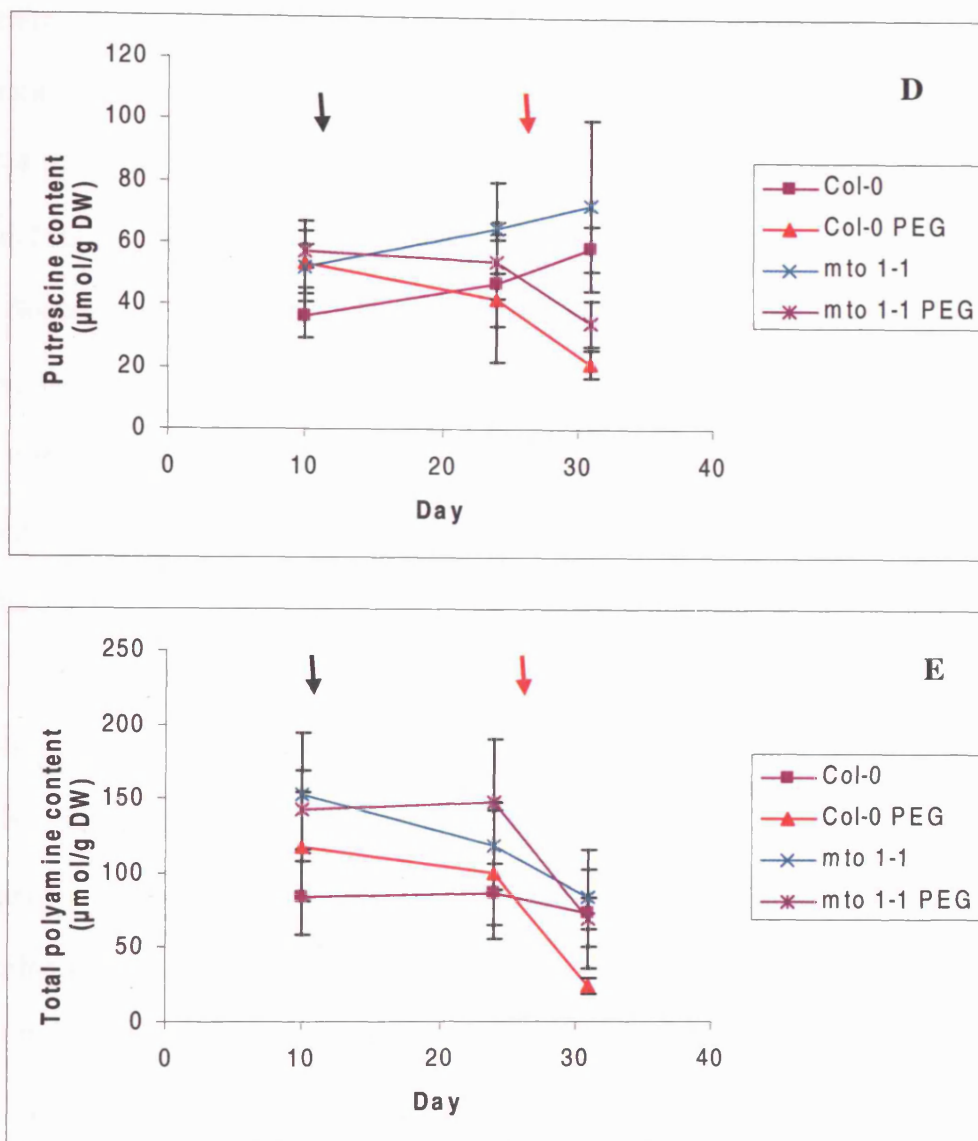
At the end of each week, the  $F_v/F_m$  was determined and one leaf was selected from each plant to monitor dry weight and the polyamine content. These were determined using the methods outlined in chapters two and three.

#### 4.4 Results

##### 4.4.1 *mtol-1*





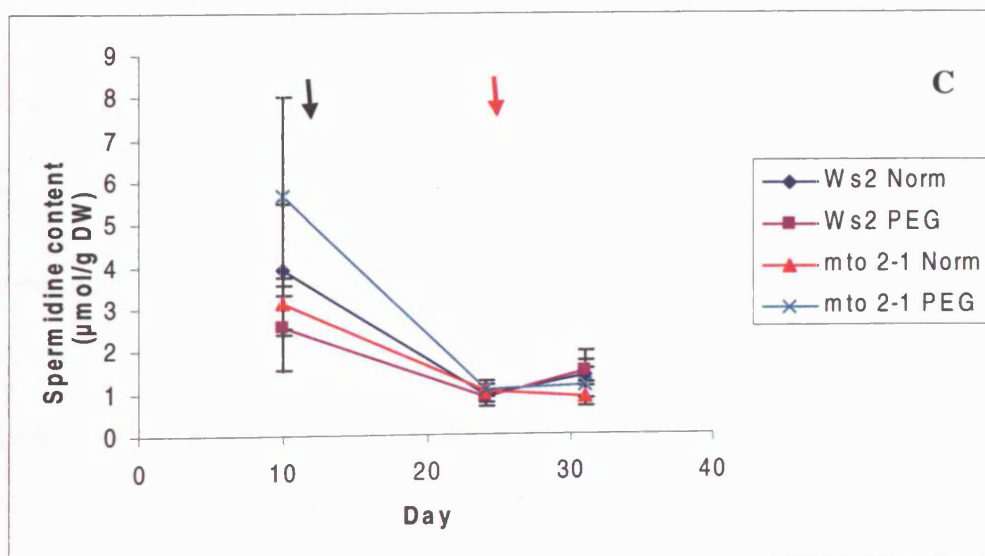
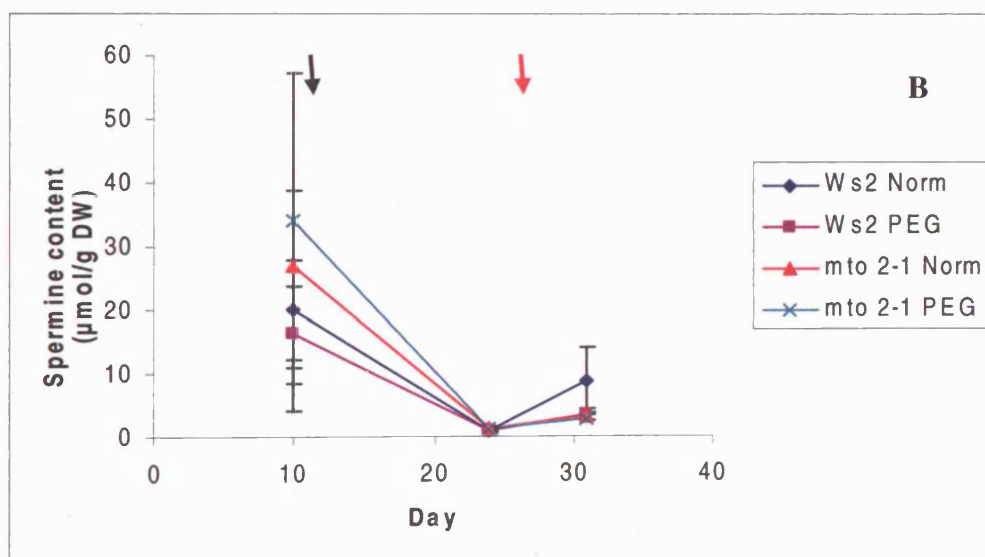
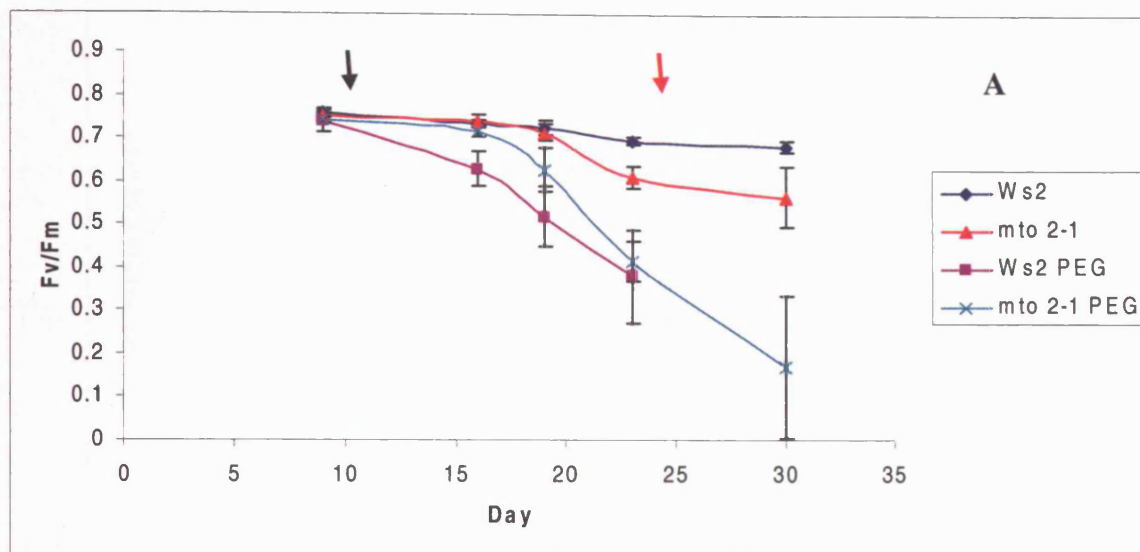


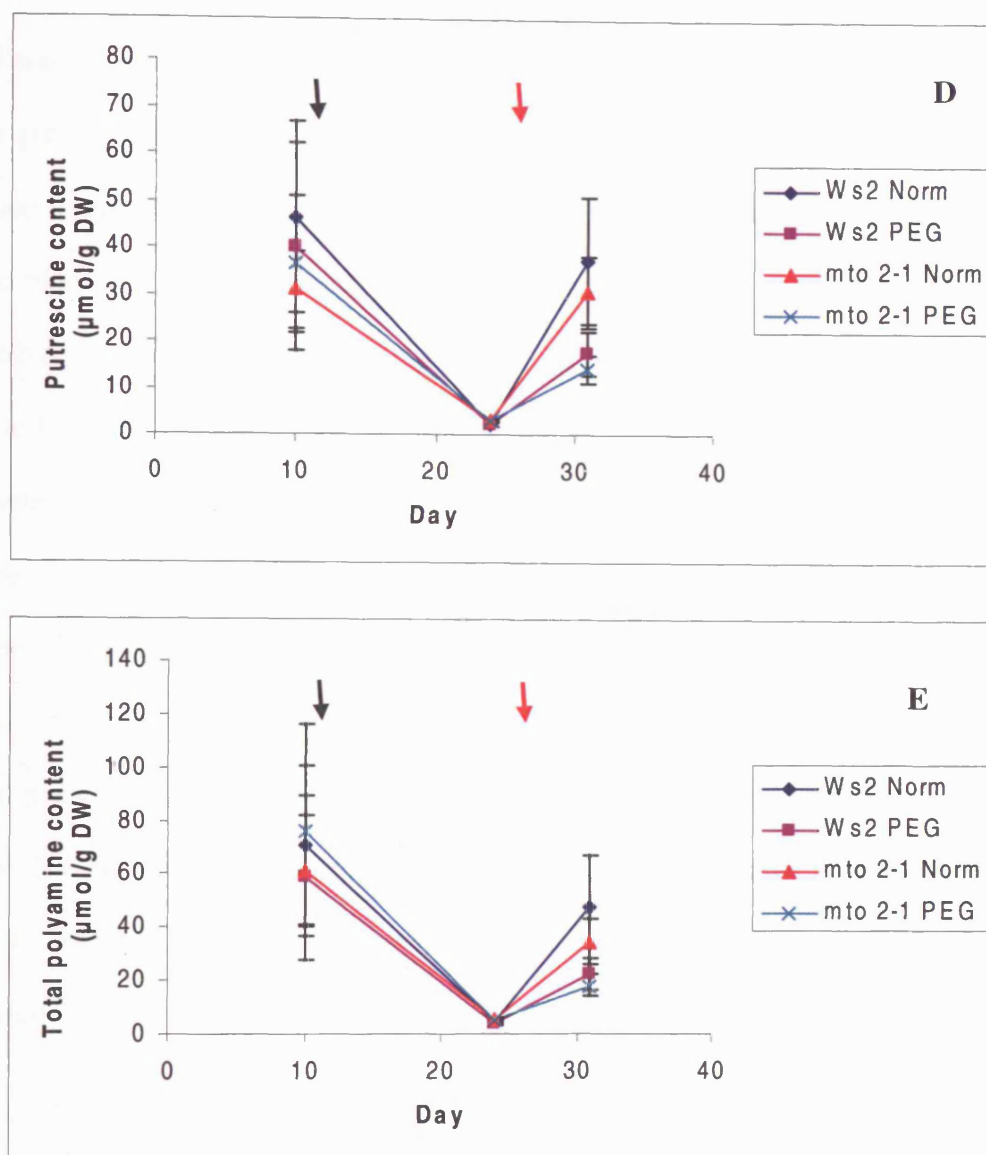
**Figure 32.**  $F_v/F_m$  (A), spermine (B), spermidine (C), putrescine (D) and total polyamine content (E) in *mtol-1* with respect to Col-0 over three weeks under osmotic stress and normal conditions (as outlined in the Materials and Methods). Error bars show standard error values. Black arrows indicate addition of 10% PEG on day 11 and red arrows indicate addition of 12% PEG on day 25.

$F_v/F_m$  was monitored every week. In this case, under normal conditions both mutant and wild type had a similar  $F_v/F_m$  throughout the experiment. However, when osmotically stressed, the plants' photosynthetic efficiency declined rapidly. The high standard error was due to variations between individual plants. There was no conclusive evidence that *mtol-1* differed in  $F_v/F_m$  from Col-0 under osmotic stress. In normal conditions, both mutant and wild type exhibited a decline in spermine content

over time (Figure 32.). The decline in the average values of spermine and spermidine over time and the increase in putrescine are possibly environmental and due to the life cycle of the plant. It has been shown that polyamines contents change before flowering Martin-Tanguy (1997). However, repeat experiments should be carried out to confirm these fluctuations in polyamines are due to the plant's life cycle and not to external factors. Osmotic stress at 10% w/v PEG did not change the rate of decline, but by week 3 and upon addition of 12% PEG, stressed wild type plants showed a sharper decline in spermine when compared to plants grown under normal conditions. Furthermore, *mtol-1* plants did not exhibit this sharp decline in spermine content. In fact, under 12% PEG stress, *mtol-1* plants had on average 15-fold more spermine than Col-0 plants (t test,  $P=0.004$ ). There was no significant change in spermidine values between the mutant and the wild type in either treatment. In normal conditions, Col-0 and *mtol-1* showed no change in the putrescine content (Figure. 28). Osmotically stressed plants had similar putrescine levels to non-stressed plants until week 3 when they decreased dramatically in Col-0 (t test between normal and PEG treated Col-0,  $P=0.005$ ) but not as much in *mtol-1* (t test between normal and PEG treated *mtol-1*,  $P=0.228$ ). There was no significant difference between Col-0 and *mtol-1* in stress conditions ( $P=0.178$ ). The total polyamine content was the same from week 1 and 2 under all conditions in mutant and wild type (Figure 32). At week 3, there was a decline in the overall polyamine content in stressed plants. There was a difference in overall polyamine content between stressed *mtol-1* and Col-0 plants in week 3 (t test  $P=0.08$ ). This was due to a difference in spermine values.

#### 4.4.2 *mto2-1*





**Figure 33.**  $F_v/F_m$  (A), spermine (B), spermidine (C), putrescine (D) and total polyamine content (E) in *mto2-1* with respect to Ws-2 over three weeks under osmotic stress and normal conditions (as outlined in the Materials and Methods). Error bars show standard error values. Black arrows indicate addition of 10% PEG on day 11 and red arrows indicate addition of 12% PEG on day 25.

Under normal conditions, there was no difference between wild type plants and *mto2-1* until week 3 where the  $F_v/F_m$  of *mto2-1* reduced. In PEG stressed plants, *mto2-1* seemed to be unaffected by 10% PEG and showed similar  $F_v/F_m$  to the plants under normal conditions. Ws-2 showed a significant decline in  $F_v/F_m$  treated with 10% PEG. After treatment with 12% PEG, the *mto2-1* plants also showed a decline in  $F_v/F_m$ . At

week 3, the  $F_v/F_m$  had declined to half its original value in both *mto2-1* and Ws-2. There was no difference in spermine and spermidine content between *mto2-1* and Ws-2 plants grown under normal conditions (Figure 33). Spermine and spermidine contents declined in all plants in week 2 and stayed at this level into week 3. In the 10% PEG-treated plants, at first it seemed that spermine content in Ws-2 was reduced more than in *mto2-1*, but this difference was not significant ( $P=0.071$ ). By week three, there was definitely no difference between mutant and wild type in either treatment. With putrescine, at week 1, there was no difference between *mto2-1* and Ws-2 (Figure 33). At week 2, all plants exhibited a decrease in putrescine. Unlike the decline in spermine and spermidine content, at week 3 there was an increase in putrescine in all plants. This increase was more pronounced in plants grown under normal conditions than in PEG-treated plants. There was no difference in overall polyamine content between *mto2-1* and Ws-2 (Figure 33). At week 2, all plants exhibited a decrease in overall polyamine content. At week 3, due to an increase in putrescine, the overall polyamine content increased.

## 4.5 Discussion

### 4.5.1 Response of *Arabidopsis* to PEG treatment.

The average polyamine content determined in this experiment is higher than previously reported in *Arabidopsis* (Table 6). Although this could be attributed to a difference in ecotype, method of growth and time of sampling it could also be that the plants are stressed after transfer onto vermiculite. It would be advisable to repeat these experiments to confirm the values shown and perhaps compare plants grown on soil to those grown on vermiculite to see if there is an additional stress. Furthermore, to confirm the values given in this thesis, a larger sample number of plants should be monitored in each experiment.

There is a high variability between plants in polyamine content even under similar conditions on day 10. This shows that polyamine content is highly variable between plants, even at a similar age. This is because even though these plants are grown in a similar environment, they differ in the time of germination, stress at transfer and even the leaf that is selected for analysis may be a slightly different age. This is why a larger population of plants should be used in the future. Furthermore, plants show a change in polyamine content under normal conditions over time. It is also clear that whilst some polyamines may increase over time others can decrease and that at any point each polyamine contents can vary. This indicates a difference in the role of each polyamine. In the Col-0 background, spermine, spermidine and putrescine were seen to decline under severe osmotic stress (12% PEG), whilst only putrescine showed a decline in the Ws-2 background. This could be as different backgrounds have been shown to have different ways of coping with PEG treatment. Interestingly, *Arabidopsis* responds to PEG differently to salt stress. Polyamines did not accumulate significantly in the leaves of any mutant or wild type under PEG stress compared to normal conditions, but they

did under salt stress. Lefevre *et al.* (2001) also observed that PEG and salt stress induced different polyamine responses in rice. They showed that putrescine and spermidine increased in ionic stresses but no increase was seen in the PEG treatment and postulated that in rice, polyamine accumulation did not require an osmotic signal and occurred at the beginning of salt stress exposure. Again, the literature gives contradictory results, as Capell *et al.* (2004) showed that PEG-treated wild type rice showed an increase in putrescine. Furthermore, *ADC2* was induced to the same extent under osmotic (mannitol) and salt stress in the AtGenExpress experiments (see Appendix). In this experiment, plants were not germinated under the stress conditions, demonstrating that *ADC2* can be induced under short-term stress. Furthermore, it was shown that the increase in *ADC2* transcript in mannitol-treated plants is faster than in salt-treated plants. Moreover, after an initial increase in response to drought stress, *ADC2* transcript was reduced to just above normal levels. This shows that different kinds of stress affect expression of *ADC2* and consequently the polyamine synthesis pathway in different ways. Therefore, it is reasonable that salt stressed plants responded by inducing polyamine synthesis whilst PEG treated plants decreased synthesis.

Why should polyamines decline under osmotic stress if they are said to be osmoprotectants? These results suggest that polyamine accumulation is linked to ionic stress. This means that polyamines may not be important in unspecific protecting roles that would benefit plants suffering from both osmotic and ionic stress, such as strengthening cell structure, or maintaining cell turgidity. If their role is specific to ionic stress, then it is likely that they are more involved with buffering excess ions or with initiating transcription of/affecting proteins that do (e.g. ion channel proteins). Liu *et al.* (2000) determined a link between stress conditions, polyamine levels, and stomatal regulation, stating that polyamines regulate the voltage-dependent inward  $K^+$

channel. It would be interesting to conduct osmotic and ionic stress experiments under the same conditions to test if polyamine accumulation really is different between these two stresses.



#### 4.5.2 *mtol-1*

The overall polyamine content in the leaves of Col-0 and *mtol-1* grown under normal conditions stayed the same over the three weeks (Figure 32). However, individual polyamines fluctuated, with spermine and spermidine gradually decreasing and putrescine showing a gradual increase. The variation of polyamine content over time is indicative of their role in plant development. Moderate osmotic stress did not change the polyamine content in either mutant or wild type. Under severe osmotic stress, the polyamine content in Col-0 declined, this was mainly due to a significant drop in spermine. Under 12% PEG stress, *mtol-1* plants had on average 15-fold more spermine than Col-0 plants. The *mtol-1* mutant did not contain more spermidine and putrescine than Col-0 (Figure 32). The increase in spermine content did not relate to a difference in  $F_v/F_m$  values, so cannot be related to a change in stress tolerance (Figure 32).

#### 4.5.3 *mtol2-1*

At week 1 there was no difference between mutant and wild type polyamine levels or  $F_v/F_m$ . At week 2, under 10% PEG treatment, the  $F_v/F_m$  of Ws-2 plants declined, whilst *mtol2-1* plants had a similar  $F_v/F_m$  compared to plants grown under normal conditions. This suggests the *mtol2-1* mutation may be important in protection against osmotic stress, though larger scale experiments need to be done. By week 3 there was no difference in  $F_v/F_m$  between mutant and wild type, perhaps as variation was higher at this stage (Figure 33). The overall polyamine content in Ws-2 decreased until week 3, where, due to an increase in putrescine, the overall polyamine content increased. This increase in putrescine was probably a physiological response, perhaps due to flowering. No link between methionine overproduction and osmotic stress tolerance was found.

The results of this chapter reflect the difference of polyamine response to different stresses, with PEG being a purely osmotic stress and salt being both an ionic and an osmotic stress. As polyamines decreased under osmotic stress, it is not surprising that there were no major differences in polyamine contents between the *mto* mutants and their wild types. Increased methionine production would only increase spermine and spermidine synthesis if SAM were limiting due to stimulation of putrescine production. It would be interesting to see what changes in polyamines occur in different tissues/treatments and to repeat the experiment on a larger scale to see if variations can be minimised. Also, it may be important to germinate seeds under osmotic stress, as the last chapter indicates that germination under stress can give different results to applying stress at a later stage.

## **Chapter Five: Screening for mutants with altered polyamine response**

## **5.1 Aim**

Aim of this chapter was to isolate *Arabidopsis* mutants that can be used to identify the pathways involved in polyamine response. To this end, a screening strategy with activation-tagged mutant lines on polyamines was designed.

## **5.2 Introduction**

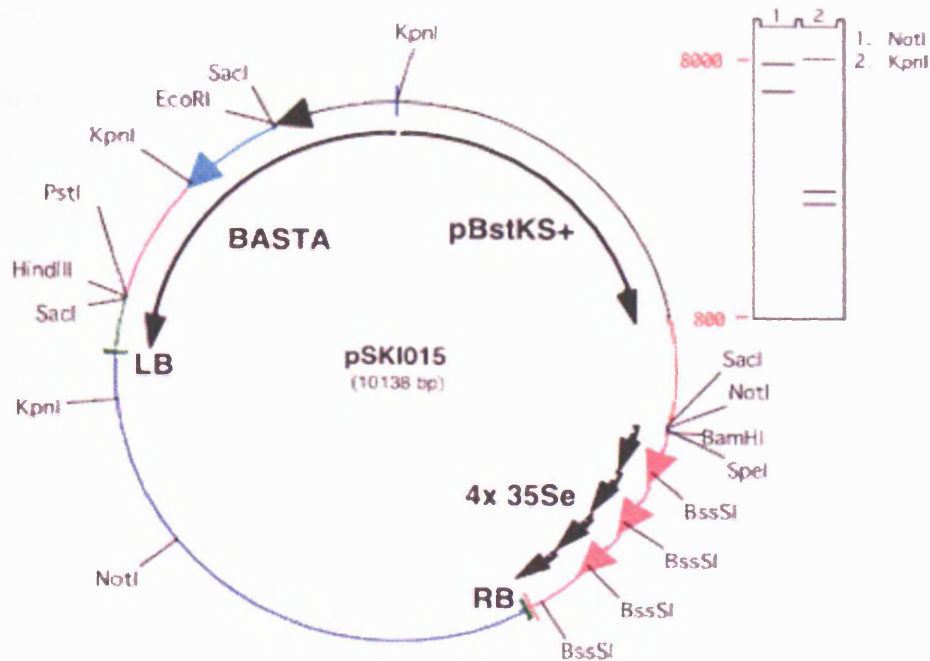
### **5.2.1 Why use *Arabidopsis* activation-tagged lines to isolate mutants?**

The *Arabidopsis* genome is organised into five chromosomes and has an size of 115,409,949 bp (Adams *et al.* 2003). It is estimated that it holds 26,828 genes of which 25,540 are annotated as protein coding (Yamada *et al.* 2003). As there is less repetitive DNA in *Arabidopsis* than in other plant genomes and as it is fully sequenced, *Arabidopsis* mutants are easier to genetically characterise than other plant mutants. In this thesis, T-DNA mutant lines were screened. As insertions are not random (Alonso *et al.* 2003), a 10-fold redundancy is assumed to be required by most scientists. This means to find a mutant in a specific gene, approximately 250,000 mutants would have to be screened.

Making mutants has always been an integral part of finding out the function and role of enzymes and compounds in a pathway (Østergaard *et al.* 1998). There are several ways researchers choose to do this, from classic mutagenesis i.e. using chemicals and radioactivity, to inserting T-DNAs and transposons. In these experiments activation-tagged T-DNA mutants were screened. With activation tagging, enhancers are incorporated in the T-DNA insert. As a result, two types of mutant are created, knock-out mutants and mutants, in which genes are over-expressed. Therefore, genes that would not be isolated by knock-out mutations, such as genes that are essential for

germination or genes whose roles are compensated for by another pathway, can be investigated. Interestingly, activation tagging can either increase the natural expression pattern of the gene affected or it could alternatively lead to constitutive overexpression. Therefore mutants made using this technique can either have a gene which is constitutively or naturally overexpressed. If the natural expression pattern is increased, the specific role of this gene will be enhanced. Activation tagging has the added advantage of creating dominant mutations. This means that the phenotype can be seen readily in the T<sub>1</sub> generation (loss-of-function mutations have to be screened in the T<sub>2</sub> generation). This is advantageous as silencing of a mutation can occur in later generations. Some activation-tagged mutants are stable up to eight generations; others can be attenuated in later generations (Weigel *et al.* 2000). Furthermore, Weigel *et al.* (2000) discussed the possibility of factors that limit the interaction of CaMV 35S enhancers with certain genes. Chung *et al.* (1993) postulated that *Arabidopsis* may contain insulator sequences that protect genes from enhancers close by, a factor which may be important in a plant whose genome is so tightly packed with genes, while Ohtsuki (1998) states that CaMV enhancers act preferably on a certain set of promoters. The pSKI015 vector (AF187951) used for activation tagging (Weigel *et al.* 2000) is outlined below, and has:

- A series of CaMV enhancers near the right border. If the enhancers are incorporated into the genome near a gene up to 3.6 kb away, expression of that gene will be increased (Weigel *et al.* 2000).
- Blue-script, which will allow for plasmid rescue and contains ampicillin resistance.
- A BAR gene, this allows mutants with the T-DNA insertion to be screened for herbicide resistance.



**Figure 34.** T-DNA insert in the Scheible and Somerville lines. Taken from <http://www.salk.edu/LABS/pbio-w/toolsfs.html>

**Key:** 35Se – Enhancers; pBstKS+ - Bluescript; BASTA – BAR gene; LB – Left Border; RB – Right Border

Mutants obtained through forward genetic screening can be examined using mapping or PCR-based techniques to locate the chromosomal location of the disrupted gene. If these methods do not succeed, more high tech methodology can be used. For example, transcript-based cloning involves analysis of gene expression using oligonucleotide microarrays to identify mutated genes in a specific mutant (Mitra *et al.* 2004). Eventually, the ultimate goal is obtain the function of the gene, which can be determined through tests on the mutant phenotype, obtaining revertants or studying complementation.

## **5.3 Materials and methods**

### **5.3.1 Plant material and growth conditions**

The activation-tagged lines used were obtained from the Nottingham *Arabidopsis* Stock Centre (Scheible and Somerville lines, Stock No. N31100). The wild type used in this study was ecotype Columbia-2 unless otherwise stated. All medium-containing vessels were bought pre-sterilised or autoclaved to avoid contamination by other organisms. Medium was autoclaved before pouring into plates in sterile conditions. If solutions could not be autoclaved (e.g. spermidine-based solutions), the solution was sterilised by filtration. Media for plant growth were created through using 1% (w/v) agar plates with half-strength Murashige–Skoog (MS) salts. For screening on spermidine, solutions were made up to 5 mM by adding spermidine solution to the medium after autoclaving.

Seeds were surface sterilised by submersion in thin bleach (Tesco) for two minutes. Samples were centrifuged briefly then washed three times in sterile distilled water. Seeds were dispersed on 1% agar plates containing Murashige–Skoog (MS) salts with and without 5 mM spermidine in sterile conditions. Plates were sealed using Micropore surgical tape. After two days in the dark cold room (4°C), seeds were grown in a growth chamber at 12-h light:12-h dark regime and 50% relative humidity at 22°C in the day and 18°C at night. Light intensity was approximately 100  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . Plates were placed vertically so that root length could be measured.  $F_v/F_m$  was measured as outlined in Chapter Two.

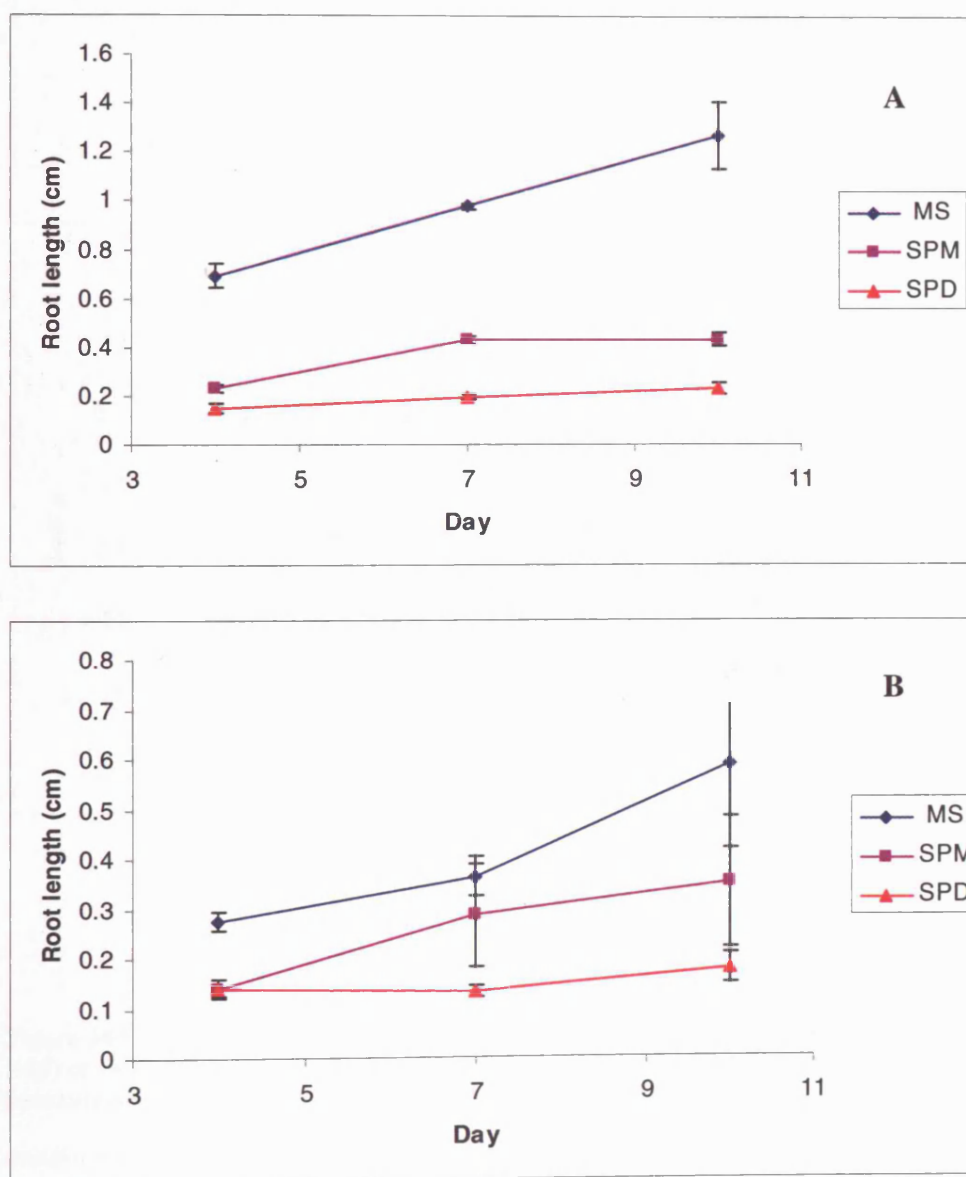
### **5.3.2 Screening for spermidine-resistant mutants**

Root length and bleaching were monitored. Plants with longer roots and greener leaf tissue than the wild type were grown for seed production and re-screened in the next generation.

## 5.4 Results

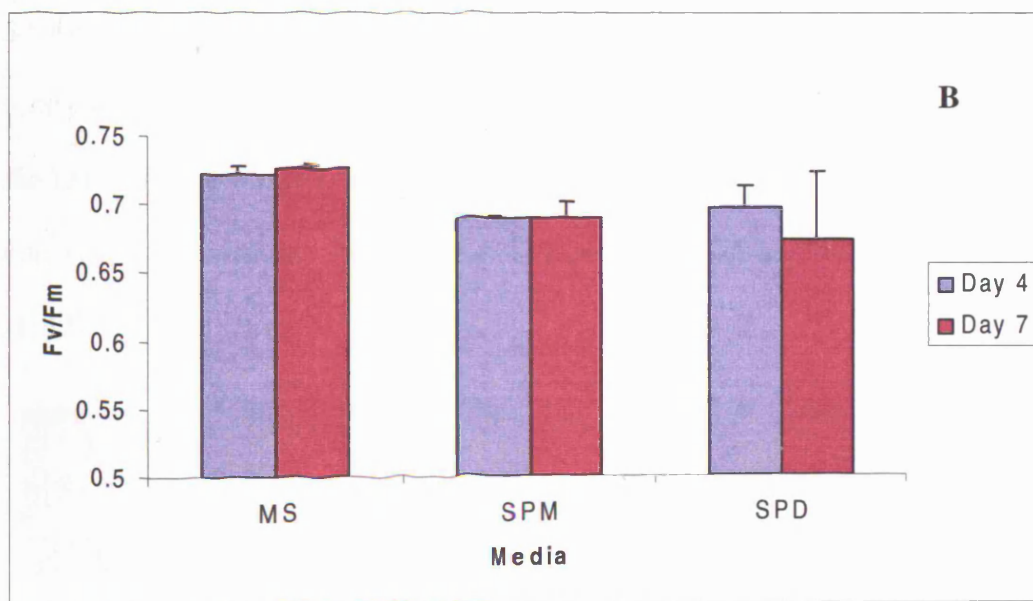
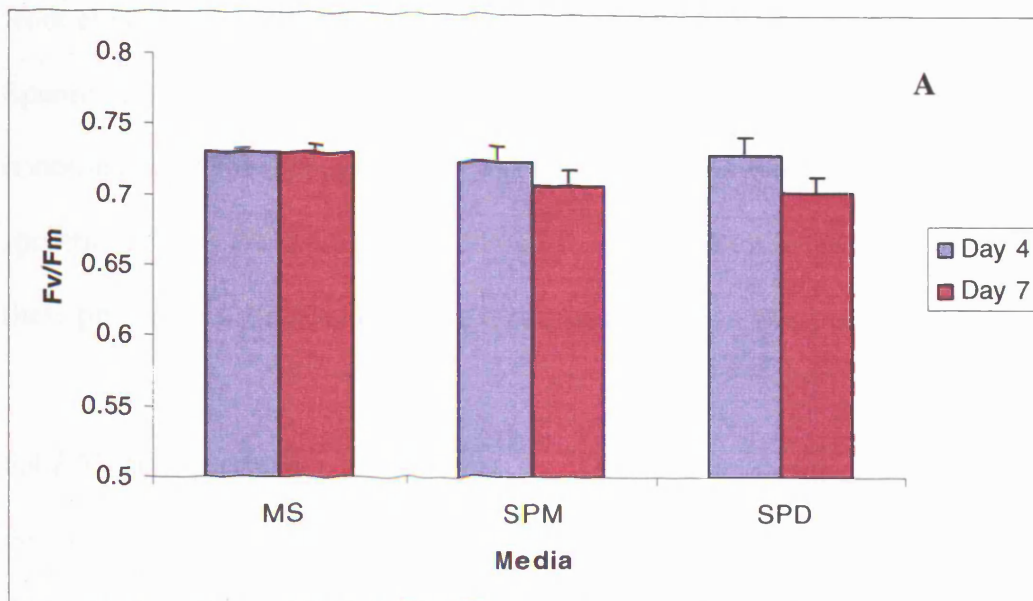
### 5.4.1 Initial experiments with wild types

To see how wild type *Arabidopsis* (Col-0 and Ws-2) grew on polyamines, root length and  $F_v/F_m$  on spermine and spermidine (5 mM) were examined. Root length taken over a range of concentrations showed that root elongation was strongly inhibited at values above 3 mM spermidine (Appendix). 5 mM was used so mutants would be clearly different from the wild-type, based on preliminary results.



**Figure 35.** Root length of Ws-2 (A) and Col-0 (B) germinated on half-strength MS medium without polyamines (MS) or with addition of 5 mM spermine (SPM) or spermidine (SPD). Mean of 20 plants. Error bars show Standard Error.



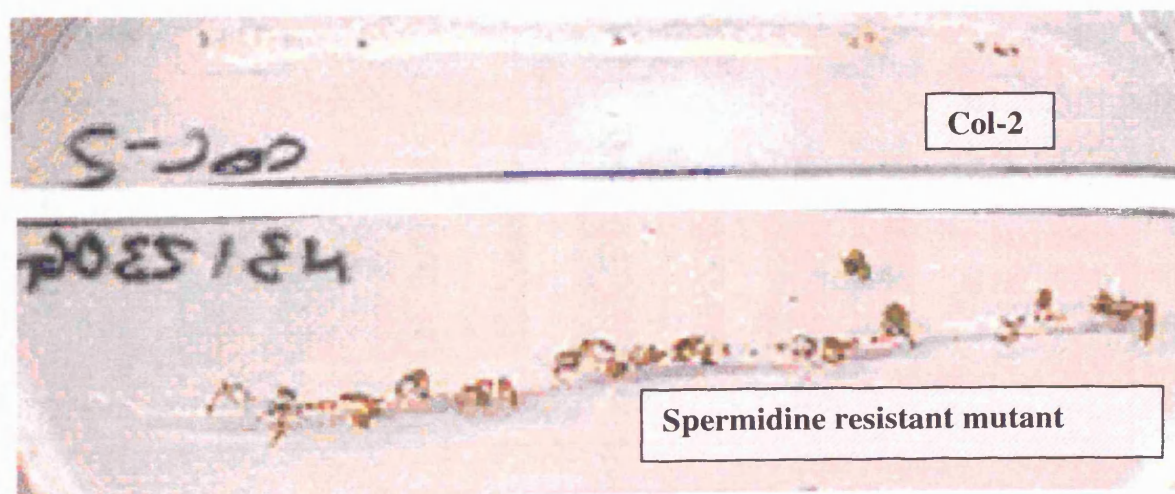


**Figure 36**  $F_v/F_m$  of Ws-2 (A) and Col-0 (B) germinated on half-strength MS medium without polyamines (MS) or with addition of 5 mM spermine (SPM) or spermidine (SPD). Mean of 20 plants. Error bars show Standard Error.

Root elongation in *Arabidopsis* was inhibited by spermine and spermidine (Figure 35). Spermidine seemed to inhibit root growth more than spermine at the same concentration. Furthermore, different ecotypes seemed to have different responses to spermidine with Ws-2 suffering a larger decrease in root length than Col-0. Growth on these polyamines did not consistently decrease  $F_v/F_m$  in Ws-2 or Col-0 (Figure 36).

#### 5.4.2 Mutant screening

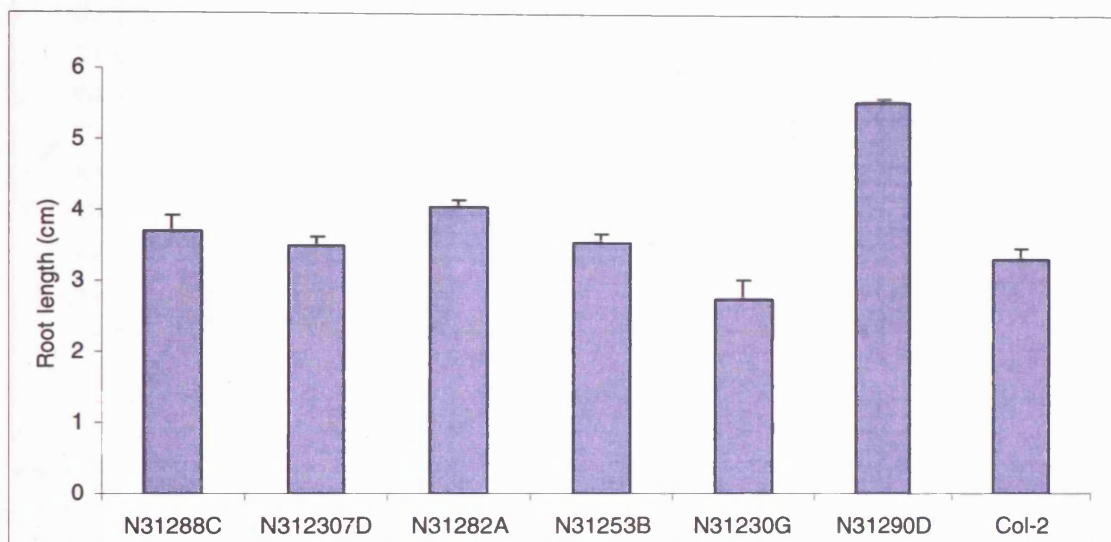
At 5 mM spermidine root growth in *Arabidopsis* was inhibited and eventually, probably due to hydrogen peroxide accumulation, plants became bleached and died. 11500 Scheible and Sommerville lines were screened on 5 mM spermidine for an increase in root length or delayed bleaching. 52 putative mutant lines were selected, grown, and the resultant seed was re-screened (Figure 37). From this, six mutant lines showed a consistent phenotype: N31290D, N31230G, N31282A N31288C, N31253B and N312307D.



**Figure 37.** Example of re-screening for polyamine response mutants.

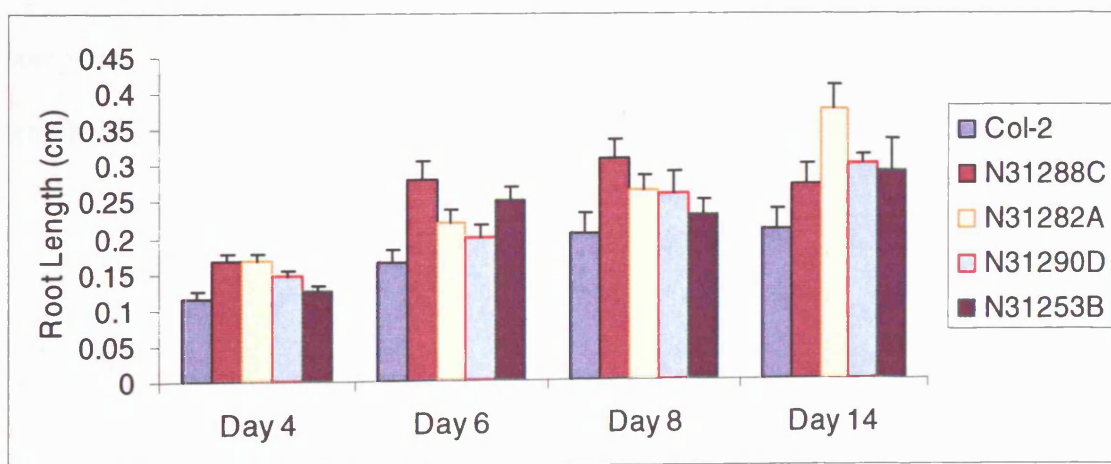
### 5.4.3 Growth on MS

All mutants grew with a similar root length to the wild type on spermidine-free medium with the exception of N31290D. This mutant grew with longer roots on MS (Figure 39).



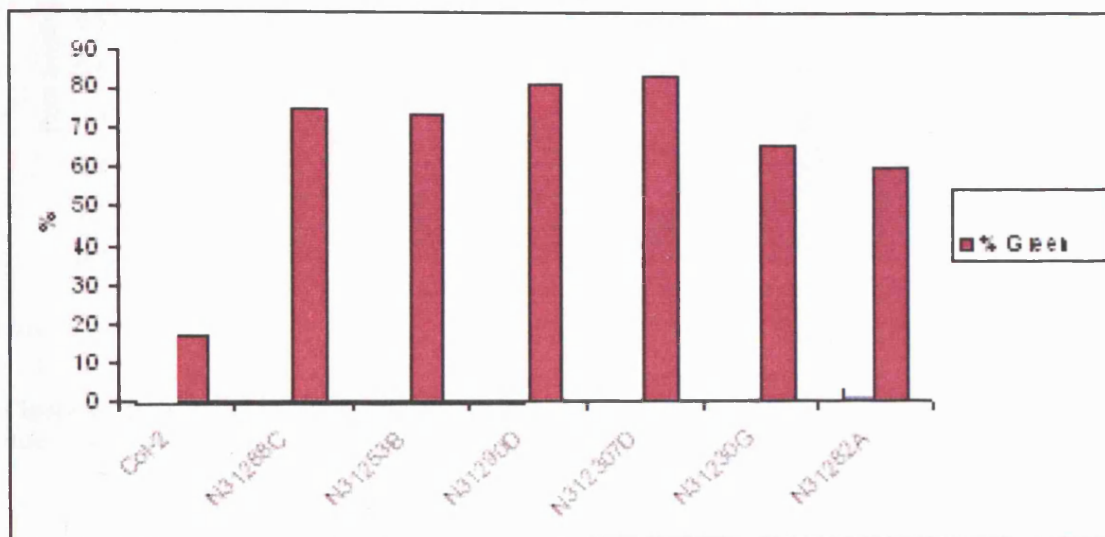
**Figure 38.** Root length on MS medium on day 14. Mean of 20 plants. Error bars show standard error.

### 5.4.4 Growth on spermidine



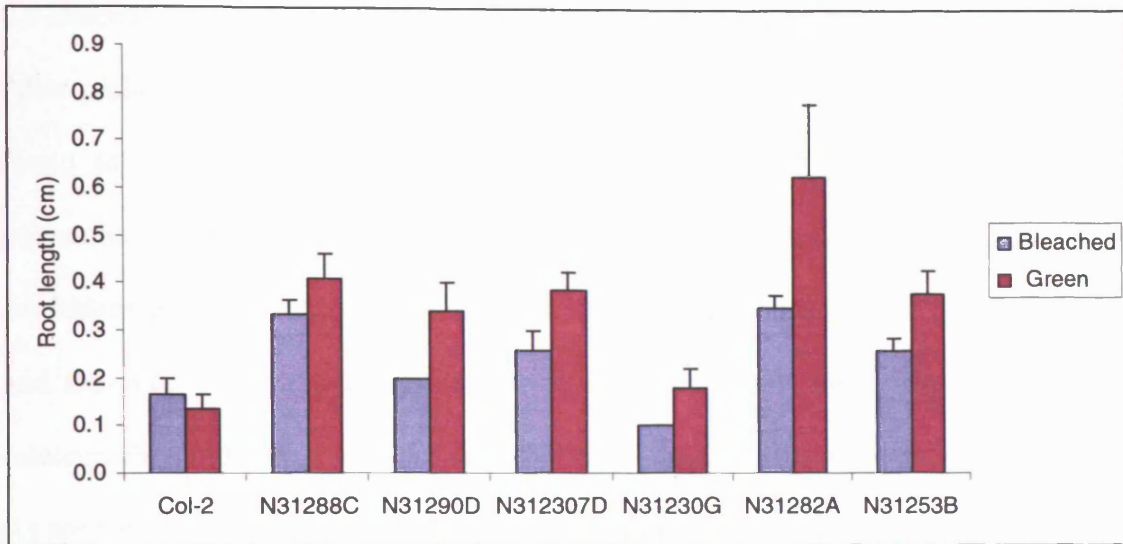
**Figure 39.** Root length MS medium supplemented with 5 mM spermidine. Mean and standard error of 20 plants.

When spermidine was added to the medium, mutants N31288C, N31282A, N31253B and N31290D had longer roots than the wild type (Figure 38). N312307D and N31230G had similar root lengths to the wild type (data not shown).



**Figure 40.** Bleaching resistance after 17 days on MS medium supplemented with 5 mM spermidine. Mean of 20 plants.

After 15 days on spermidine-supplemented medium, the N31290D, N31288C, N31282A, N31230G, N312307D and N31253C mutants showed decreased bleaching compared to the wild type (Figure 40). To look further at bleaching in the mutants, root length of bleached and green plants was measured.



**Figure 41.** Root length of bleached and green plants grown on spermidine-supplemented medium for 17 days. Mean and standard error of 6-20 plants.

In the wild type the root length of bleached and green plants was similar (Figure 41).

On average, green mutant plants had longer roots, indicating that root length and non-bleaching characteristics may be linked (Figure 41).



## 5.5 Discussion

After preliminary studies in this chapter, root length and resistance to bleaching were found to be an effective screen for spermidine-resistant plants. The presence of polyamines in the growth medium inhibited root elongation and caused bleaching in *Arabidopsis*. The extent of root inhibition is dependent on the type of polyamine applied and the ecotype of the plants tested. It was clear from these results and previous research that individual polyamines were differentially involved in root architecture. As spermidine gave clearer results than other polyamines, MS plates supplemented with 5 mM spermidine were used to screen for mutants. Effects of polyamines on  $F_v/F_m$  were variable. Furthermore, as measuring  $F_v/F_m$  was a laborious process, screening for root length and resistance to bleaching was used to look at the resistance of the mutant lines.

Although it was spermidine that was applied exogenously, it cannot be postulated that it was this compound that was affecting plant growth. Tassoni *et al.* (2000) reported that spermidine-treated *Arabidopsis* converts excess spermidine into putrescine in seedling cotyledons. Spermidine, spermine and agmatine showed a positive correlation with primary root length of cabbage (Hummel *et al.* 2004) and putrescine was shown to have negative or null effects. Therefore, mutants isolated from screening on 5 mM spermidine could include mutants affected in the transport, metabolism or storage of any of the polyamines.

Two types of mutant were isolated from the spermidine screen:

1. Mutants with delayed bleaching, but without increased root length.
2. Mutants with both delayed bleaching and increased root length.

A summary of the characteristics of each mutant is listed in Table 10.

**Table 10.** Summary of mutant characteristics.

Mutant	Increased root length on MS	Under spermidine-supplemented conditions	
		Increased root length	Delayed bleaching
N31290D	Yes	Yes	Yes
N31288C	No	Yes	Yes
N31253B	No	Yes	Yes
N31282A	No	Yes	Yes
N31230G	No	No	Yes
N312307D	No	No	Yes

Apart from decreased bleaching and increased root growth, the mutants showed no additional developmental or floral abnormalities. This contrasts with mutants isolated on spermine by Mirza and Saeed (1997), which had abnormalities such as an increased production of cauline leaves and branches.

## **Chapter Six: Genetic characterisation of mutants with altered polyamine response**



## **6.1 Aim**

Aim of this chapter was to determine the genetic background of the spermidine-resistant mutants isolated from screening activation-tagged lines. The mutants were back-crossed to the wild type Col-2. An F<sub>2</sub> population was isolated and screened on MS medium supplemented with spermidine or BASTA. Only N31290D, N31288C and N31282A were successfully backcrossed, therefore, due to time constraints only these were analysed.

## **6.2 Introduction**

By examining the F<sub>2</sub> generation, the type of mutant can be determined. If the ratio of mutant to wild type phenotype is 3:1 in the F<sub>2</sub> generation, the mutation is dominant. If it is 1:3 then the mutant is recessive. A 3:1 ratio was expected for mutants with enhanced gene expression. However, if the T-DNA had disrupted a gene, then either a 3:1 or a 1:3 ratio may appear. 2:1 ratios may occur if a homozygous mutant is lethal.

Furthermore, on BASTA-supplemented medium, if the mutant has one T-DNA insertion, a 3:1 ratio of resistant to susceptible plants would be seen. If there is more than one T-DNA insertion in unlinked chromosomal locations, then more resistant plants would be seen. As BASTA resistance is dominant, a ratio lower than 3:1 was not expected.

## **6.3 Materials and Methods**

### **6.3.1 Growth conditions**

Screening on MS and spermidine was outlined on Chapter Five. For screening on BASTA, two methods were tried. Firstly, mutant plants were grown on soil and sprayed with 0.002% w/v glufosinate-ammonium. This proved to be time consuming and inconsistent. Finally, 0.5 ml of 1% w/v glufosinate-ammonium was added to 500 ml of medium after autoclaving and before pouring plates.

### **6.3.2 Backcrossing**

*Arabidopsis* flowers consist of four whorls of organs, an outer whorl of four sepals then four petals, then six stamens, and finally two carpels. The first three whorls were removed, leaving the female reproductive organs. The best results were obtained when moist stigmas from the wild type were fertilized using sticky yellow pollen from the mutant plant. Col-2 was used as the female plant so that it would be easy to see if cross-fertilisation was unsuccessful, as the progeny would not be BASTA resistant.

### **6.3.3 G Test**

This tests the probability of the observed ratio differing from the expected ratio. If a P value of below 0.05 is observed, then the ratios are different. Therefore, the closer the P value is to 1.0 the more likely the ratio expected is correct.

## 6.4 Results

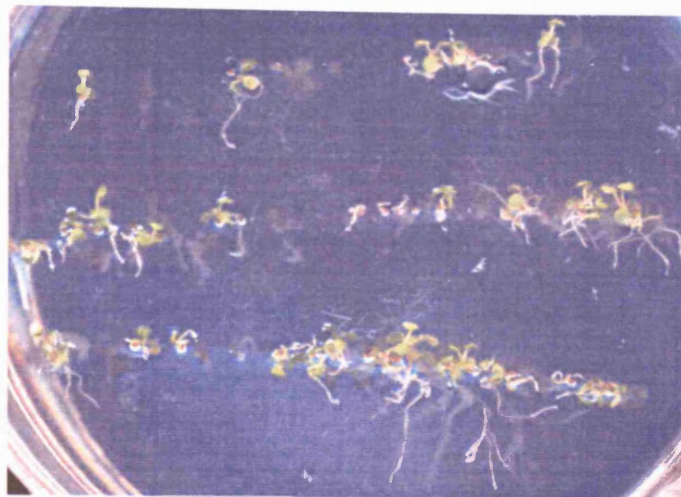
### 6.4.1 N31290D

This mutant grew with longer roots on MS and on spermidine-supplemented medium (Figure 38, Figure 39). N31290D was successfully backcrossed to its parent line Col-2, using pollen from the mutant. F<sub>2</sub> plants were screened both on media supplemented with spermidine or the herbicide BASTA. The comparison of segregation patterns on both media was used to determine whether the BAR gene in the T-DNA segregated in a similar fashion to the mutant phenotype. As expected, there was approximately a 3:1 ratio of BASTA-resistant to sensitive plants in the F<sub>2</sub> generation (t test P=0.33).

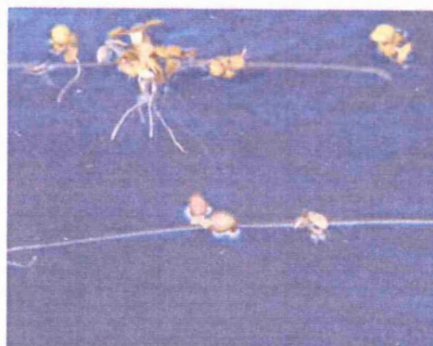
Such a clear ratio was not seen with plants screened on spermidine (Figure 42). Increased root length is not a clear phenotype and if the mutation is co-dominant then it is hard to differentiate between mutant roots (0.4 cm and above), wild type roots (0.2 cm) and heterozygous plants (in between 0.2-0.4 cm).

Yet, when plants were separated into the groups above, there seemed to be a 1:2:1 ratio (G test, P=0.82), indicating that this long root mutant was co-dominant.

A

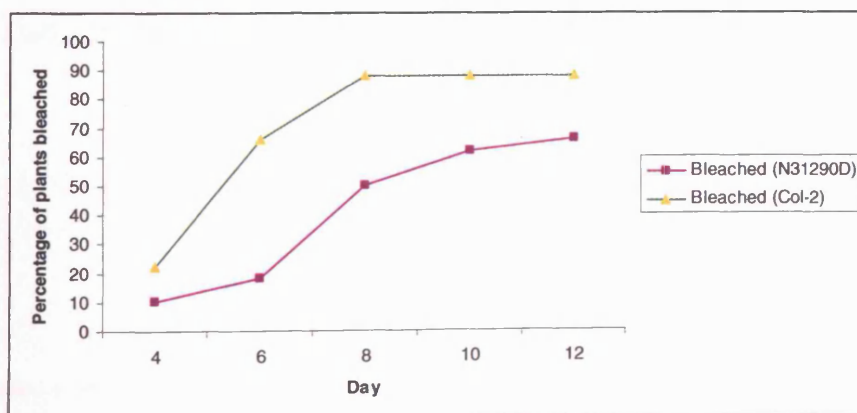


B



**Figure 42.** F<sub>2</sub> from backcrossed N31290D seeds grown on MS medium supplemented with the herbicide BASTA for twelve days (A) and on spermidine-supplemented plates (B).

The ratio of green plants to bleached plants changed over time in the F<sub>2</sub> generation (Figure 43).

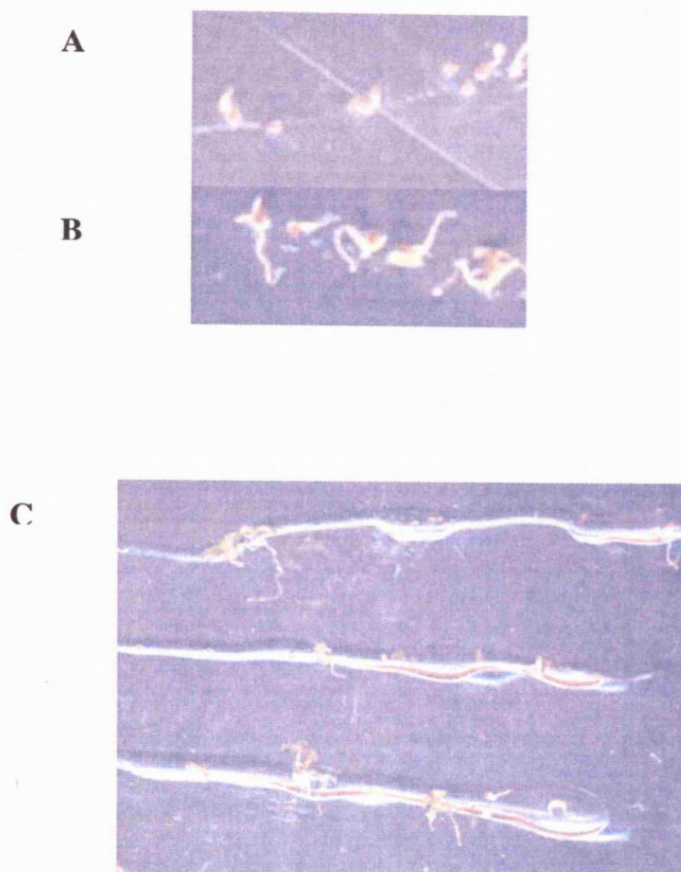


**Figure 43.** Percentage of plants bleached when grown on spermidine.

It is clear that N31290D showed delayed bleaching (Figure 43). At day 6, there were 63 green mutant plants and 14 bleached plants, this showed a 3:1 ratio. Yet, by day 12 the mutant had 26 green and 51 bleached plants which gave a 3:1 ratio, bleached to green plants (G test  $P=0.08$ ), however a 2:1 ratio fitted better ( $P=0.93$ ). In contrast, 88% of Col-2 plants were bleached at day 12. N31290D eventually reached 88% bleaching by day 27.

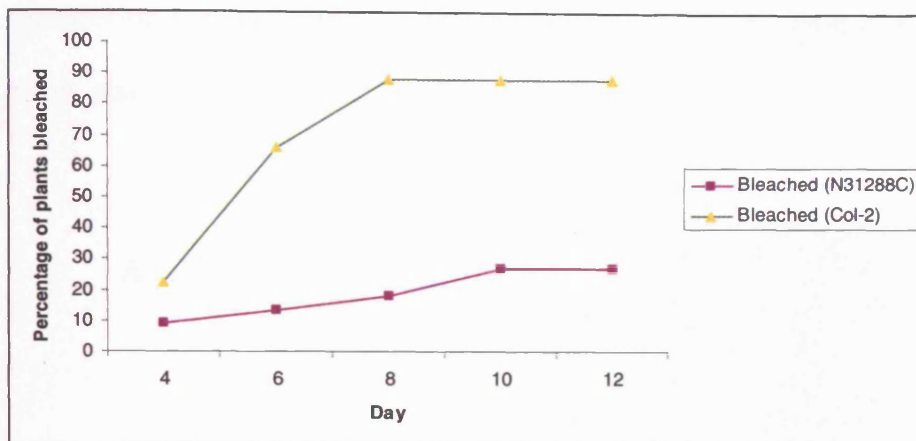
#### **6.4.2 N31288C**

N31288C had a similar root length to Col-2 when grown under normal conditions. Furthermore, this mutant showed delayed bleaching when grown on 5 mM spermidine. The mutant had a larger root length than the wild type on BASTA, but was not as resistant as other mutants (Figure 44). Perhaps the mutation was not connected to the T-DNA, but allows for a longer root length on BASTA-supplemented medium anyway. Alternatively, the BASTA gene could be repressed by the mutation. A 3:1 ratio was observed for the ratio of long to short roots on BASTA-supplemented medium (G test,  $P=0.39$ ), but a 2:1 ratio was more likely (G test,  $P=0.81$ ). Overall, screening the  $F_2$  generation of N31288C on spermidine gave a 1:3 ratio, long roots to short roots (G-test,  $P=0.81$ ).



**Figure 44.** Growth on BASTA-supplemented medium after twelve days; Col-2 (A), N31288C (B). N31288C grown on spermidine-supplemented medium for twelve days (C).

As seen in Figure 45, as well as increased root length, N31288C clearly exhibited delayed bleaching. At day 6, the majority of Col-2 plants began to bleach. In contrast the majority of N31288C plants did not begin to bleach until day 12. By day 10, when most Col-2 plants were bleached, 75% of N31288C plants were still green. This relates to the 3:1 ratio expected (G value,  $P=0.81$ ).



**Figure 45.** Comparison of bleached plants in N31288C and Col-2 when grown on spermidine-supplemented medium.

Interestingly, when N31288C plants were grown to isolate homozygous lines, some plants had an abnormal phenotype, with the inflorescence stems looping over each other instead of growing to the light and no seed production (Figure 46).



**Figure 46.** Stunted phenotype of possible homozygous N31288C plant compared to Col-2.

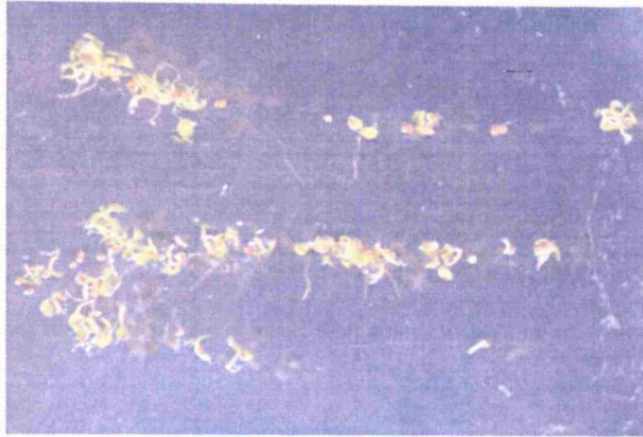
### 6.4.3 N31282A

The N31282A mutant had a similar root length to Col-2 under normal conditions. On spermidine-supplemented medium, the mutant exhibited delayed bleaching and



germination (see Appendix). On BASTA (Figure 47), the ratio of resistant to sensitive plants was 3:1 ratio (G test,  $P=0.91$ ).

A



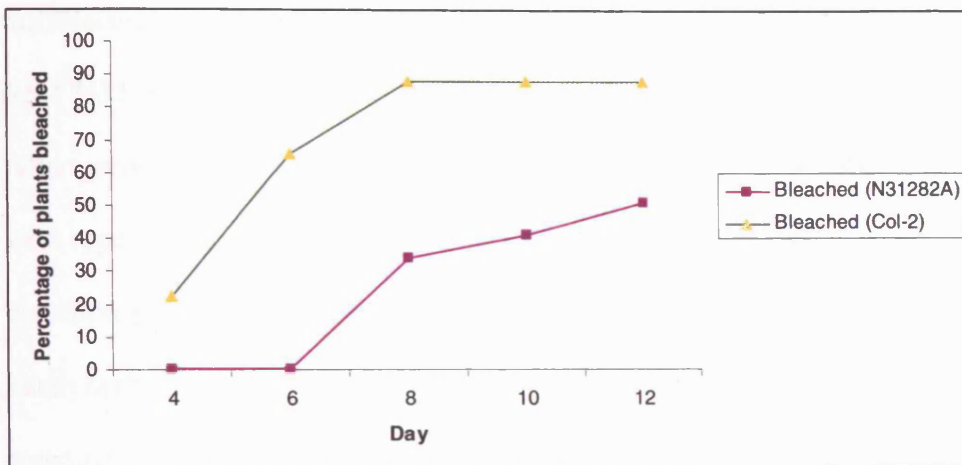
B



**Figure 47.** N31282A grown on BASTA-supplemented medium (A), N31282A grown on spermidine-supplemented medium (B).

After 12 days growth on spermidine, the  $F_2$  generation of N31282A showed a 1:1 ratio of long:short roots ( $P=0.37$ ) (Figure 47). At day 8, most Col-2 plants had bleached (88%) whilst only 21 of 61 N31282A  $F_2$  plants had bleached. This gives a 2:1 ratio green to bleached plants (G test,  $P=0.8$ ) or a weak yet significant 3:1 ratio ( $P=0.1$ ) (Figure 48).





**Figure 48.** Comparison of bleached plants in N31282A and Col-2 when grown on spermidine-supplemented medium.

## **6.5 Discussion**

### **6.5.1 N31290D**

After growth on spermidine-supplemented medium, mutant plants were greener than wild type plants. In the F<sub>2</sub> generation at day 6, there was a 3:1 segregation of green to bleached plants, the probability for this value was significant, but the probability was much higher for a 2:1 ratio (Figure 42). Perhaps the homozygous mutation was lethal in some conditions. Yet, by day 12 the likely ratio was 1:3, green to bleached plants. If the N31290D mutant is co-dominant, the heterozygous plants, whilst having delayed bleaching, would have bleached before the homozygous mutant plants. In other words, a 1:2:1 ratio of bleached to slightly delayed bleaching to delayed bleaching plants would be seen. This could explain why, by day 12, the ratio of green to bleached plants changed from 3:1 to 1:3.

Root length data also suggested a 1:2:1 ratio, although it was difficult to decide which plants are homozygous or heterozygous based on root length alone. BASTA resistance segregated in the F<sub>2</sub> generation giving a clear 3:1, resistant:non-resistant phenotype.

### **6.5.2 N31288C**

This mutant showed delayed bleaching and extended root length when grown on spermidine. The F<sub>2</sub> generation showed a 3:1 ratio of green to bleached plants, indicating that the mutation was dominant for the non-bleaching characteristic (Figure 45). The F<sub>2</sub> generation had a 1:3 ratio of long to short roots. This shows that the mutation for long roots on spermidine is recessive and that a gene has probably been disrupted. It is possible that more than one T-DNA has inserted into this mutant.

BASTA resistance was not clear in this mutant, although the mutant had longer roots than the wild type (Figure 44). This could be for two reasons. The mutation may disrupt the ability of the BAR gene to cause resistance to BASTA or the mutation may allow for longer root growth on BASTA-supplemented medium without the aid of the BAR gene (in other words the mutant does not contain a complete T-DNA construct). The F<sub>2</sub> generation segregated on BASTA with a 3:1 ratio (P=0.39) resistant to susceptible plants, however, a 2:1 ratio (P=0.81) fits the segregation pattern better. Perhaps a homozygous mutation was lethal under these conditions. Furthermore, when some F<sub>2</sub> plants were grown, a stunted phenotype was observed (Figure 46). It would be interesting to test whether these plants were homozygous.

### **6.5.3 N31282A**

Delayed bleaching, germination and increased root length were apparent in this mutant with comparison to Col-2. There was a 3:1 ratio of resistant to susceptible plants on BASTA. However, on investigation of spermidine-resistant plants no clear 3:1 ratio was seen with increased root length. Instead, a 1:1 ratio was most likely (Figure 47). There was a 3:1 ratio of green to bleached plants indicating a dominant non-bleaching mutant, however the probability for a 2:1 ratio was much higher (Figure 48). Perhaps the homozygous mutant was lethal in some conditions. The mutant showed delayed germination, although there was no correlation between delayed bleaching and root length.

#### 6.5.4 Summary of mutant segregation patterns

Table 11 summarises the results. A clear 3:1 segregation on BASTA-supplemented medium showed that the T-DNA has inserted into one point in the chromosome, or that multiple inserts have occurred in a linked chromosomal location.

**Table 11** Summary of results of segregation analysis

Mutant	Segregation in spermidine-supplemented medium	Segregation in BASTA-supplemented medium
N31290D	3:1 - BL (P=0.08) 2:1 - BL (P=0.93) 1:2:1 - RT (P=0.83)	3:1 (P=0.33)
N31288C	3:1 - BL (P=0.81) 1:3 - RT (P=0.81)	3:1 (P=0.39) Although not as resistant as other mutants
N31282A	3:1 - BL (P=0.1) 2:1 - BL (P=0.8) 1:1 - RT (P=0.37)	3:1 (P=0.91)

**Key:** BL – green to bleached plant ratio, RT – long:short root ratio

If spermidine resistance is caused by the activation tag, then it would follow that the segregation pattern for plants grown on BASTA and spermidine-supplemented medium would be the same. However, this will not be the case if the mutant is recessive (i.e. caused by gene disruption and not enhancement) or if the mutation is unlinked to the T-DNA. This is a possibility as the frequency of actual gene tagging remains low and the incidence of mis-segregation events can be more than 60% of the total mutants observed in some populations. This is as T-DNA integration can cause DNA “irritation” (a type of unforced mutation that is not linked with the gene tag), which can result in phenotypes that do not segregate with the T-DNA insert (Hayashi *et al.* 1992). In retrospect, it would have been better to obtain segregation patterns on BASTA and spermidine to confirm whether the mutation was also linked to BASTA resistance. To do this, 20 F<sub>2</sub> plants could be selected, grown to the F<sub>3</sub> generation and then screened on spermidine and BASTA. The original genotype of the F<sub>2</sub> plant can be determined (homozygous mutant, heterozygous or wild type for both spermidine and BASTA

resistance). The reason to use this more laborious process is that plants cannot be transferred from spermidine to BASTA mediaum and furthermore, with variable phenotypes like root length it is difficult to determine spermidine resistance when comparing one plant from the F<sub>2</sub>. By examining its progeny, it can be clear whether this plant is homozygous, heterozygous or mutant and therefore get a clearer idea of the segregation ratio. In retrospect, it would have been interesting to obtain segregation patterns on BASTA and spermidine. To do this, twenty F<sub>2</sub> plants could be selected, grown to the F<sub>3</sub> generation and then screened on spermidine and BASTA. The original genotype of the F<sub>2</sub> plant can be determined (homozygous mutant, heterozygous or wild type for both spermidine and BASTA resistance). This information can be used to see if BASTA and spermidine resistance is linked. It is interesting that some, possibly homozygous progeny of N31288C has growth defects. This is a common trait amongst polyamine mutant plants, as polyamines are important in many growth processes. For instance the *ADC* genes are essential for seed growth (Urano *et al.* 2005) and furthermore, mutants in SPMS have been related to defects in the elongation of stem internodes (Hanzawa *et al.* 2000). The N31288C mutant stems do not grow toward the light like the wild type. This could be due to a structural problem in the stem or that N31288C has a phototropism-related mutation. The N31288C mutant did not give seed, this could be due to an increase in spermidine uptake as an increase in polyamines content in plant ovaries has been linked to induction of parthenocarpy (Fos *et al.* 2003).

## **Chapter Seven: Identification of insertion sites of T-DNA in the polyamine response mutants**

## **7.1 Aim**

Aim of this chapter was to identify the sequence next to the site of T-DNA integration by PCR based techniques.

## **7.2 Introduction**

As stated in Chapter Six, the Scheible and Sommerville lines contain the pSKI015 vector. This allows the researcher to adopt several methods to determine which gene is adjacent to the insert. Three methods were used in this thesis.

1. Adaptor PCR
2. Plasmid Rescue
3. Inverse PCR

### **7.2.1 Adaptor PCR**

This technique (Figure 49) relies on digesting the mutant genomic DNA using blunt end enzymes that often cut in *Arabidopsis* DNA, but do not cut within primers corresponding to the T-DNA sequence. The resulting fragments are then ligated to an adaptor. PCR is performed using primers binding to the T-DNA and the adaptor sequence. An amine group on the lower strand of the adaptor stops the extension of the 3' end of adaptor-ligated genomic fragments, preventing the amplification of DNA that does not include any T-DNA sequence. The primary PCR mix is diluted and secondary PCR is done using nested primers. This should isolate DNA flanking the T-DNA insert. This method is reliant on the enzymes not cutting too far away from the end of the T-DNA, so as to prevent PCR of a fragment. As the sequence is unknown, a variety of enzymes are used.

#### 1. Genomic DNA

#### 2. Digest

#### 3. Adaptor

#### 4. PCR



**Figure 49.** Outline of Adaptor PCR, T-DNA insert in light blue, adaptor in dark blue, primers indicated by arrows.

#### 7.2.2 Inverse PCR

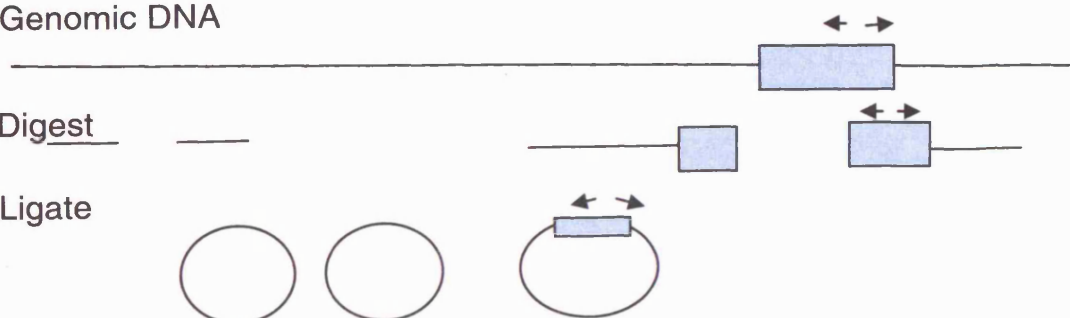
Here, primers are designed at the end of the T-DNA pointing away from each other. DNA is digested with enzymes that cut close to these primers but not between them and the end of the insert (Figure 50). The DNA is ligated and a PCR performed. A PCR product should be obtained, providing the primers are not too far apart. If successful, the resulting band would give the sequence of the end of the insert and the *Arabidopsis* DNA adjacent to it.

#### 1. Genomic DNA

#### 2. Digest

#### 3. Ligate

#### 4. PCR

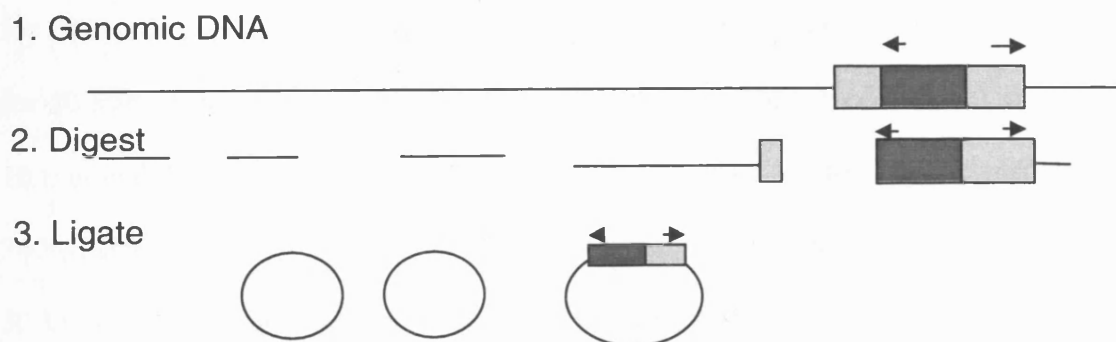


**Figure 50.** Outline of inverse PCR, T-DNA insert in light blue, primers indicated by arrows.



### 7.2.3 Plasmid Rescue

The pSKI015 fragment has bluescript (pBstKS+) within it. Enzymes that cut once on the right side of bluescript (left border plasmid rescue) or the left side of bluescript (right border plasmid rescue) and leave bluescript intact are used. The resulting fragment is ligated and this plasmid can be transformed directly into bacteria for cloning. Colonies are selected for ampicillin resistance and by using primers from the relevant border and the T3/T7 primers located at the ends of the bluescript sequence, the DNA flanking the insert can be isolated. In Figure 51, right border plasmid rescue is outlined, the primers used would be T3 from bluescript and the right border primer designed at the end of the T-DNA insert (RB1).



**Figure 51.** Outline of right border plasmid rescue. T-DNA insert in light blue, bluescript in dark blue., primers indicated by arrows.

## 7.3 Materials and Methods

### 7.3.1 Isolation of genomic DNA from *Arabidopsis thaliana*

DNA was isolated from 12 plants per mutant from the same generation that was used for re-screening and pooled. DNA was isolated from leaf tissue as described by Carroll *et al* (1995). Plant leaf tissue totalling 1 g (fresh weight) was immediately frozen in liquid nitrogen. Plant tissue was ground in liquid nitrogen and the powdered tissue mixed with 3 ml of extraction buffer (0.2 M Tris-HCl pH 7.5, 0.05 M EDTA, 2 M NaCl, 2% w/v hexadecyltrimethyl ammonium bromide) and 1 ml 5% w/v SDS. The extraction mixture was incubated at 60°C for 20 min and then mixed with an equal volume of phenol:chloroform:isoamyl alcohol 12:12:1 (Qiobiogene). This mix was centrifuged for 20 min at 340 g to separate the phases. 4 ml of the aqueous phase was mixed with an equal volume of cold isopropanol and the nucleic acids left to precipitate for 15 min at room temperature. The precipitate was pelleted by centrifugation at 340 g for 30 min. The pellet was washed once in cold 70% ethanol, centrifuged at 340 g for 10 min and the supernatant removed. The pellet was then air-dried before suspension in 200 µl sterile distilled water. DNA was examined on an agarose gel. DNA bigger than 50 kb, with a concentration of 1 µg/µl and which showed minimum smearing was used for the next step. If DNA was dirty (showed smearing), 500 µg of phenol/SEVAG was added to the sample. After briefly vortexing at a low speed, samples were centrifuged. 1/10 (of the volume of sample) of sodium acetate (3 M) and 2X (the volume of the sample) ethanol were added. Samples were centrifuged again and the supernatant was removed. The pellet was dissolved in 400 µl of sterile water and re-checked on a 0.7% (w/v) agarose gel.

### **7.3.2 Removal of RNA from DNA**

The DNA solution was increased to 2 ml by addition of sterile distilled water. 5 µl of RNase-It™ Ribonuclease Cocktail (RNase A and RNase T1) (Stratagene) was added to the mix at 37°C for 15 min. The digest was mixed with an equal volume of phenol:chloroform:isoamyl alcohol 12:12:1 (Qiobiogene) and centrifuged at 340 g for 20 min. The DNA was precipitated by mixing the aqueous phase with 266 µl of 3 M sodium acetate and 5 ml cold 100% ethanol. The precipitate was pelleted by centrifugation at 340 g for 10 min and washed with 70% cold ethanol. The pellet was then air-dried before suspension in 200 µl sterile distilled water and the DNA examined on an agarose gel.

### **7.3.3 Determining the amount and purity of DNA**

Quantity of DNA was calculated by using the fact that 50 µg/ml of DNA gives an absorbance of 1 at 260 nm (Sambrook *et al.* 1989). Purity was determined through calculating the Abs260/Abs280 ratio. DNA with minimal protein impurity will give a ratio at about 1.8. 5 µl of DNA isolated was diluted in 495 µl sterile water. A spectrometer (UV 1101 Biotech photometer, Biolabo, SA) was used to measure the absorbance of these samples at 260 and 280 nm compared to a blank of 500 µl sterile water.

### **7.3.4 Primers**

The following left (LB) and right (RB) border primers were used. Primers were designed a little before where the right border primers (RB) and left border primers (LB) were thought to be (according to the features listing on the NCBI site (AF187951)).

RB1 – CTATCGTTCAAGATGCCTCTACCGACAGTG

RB2 – GACGTTCCAACCACGTCTTCAAAGCAAGTG

LB1 –CAAACCTTGACAGTGACGACAAATCGT

LB2 - CGATATCTAGATCTCGAGCTCGAGATC

LB3 – GCGGACGGCCAA TAC TCA ACT TCA AGG

LB4 – GCTGATCCATGTAGATTTCGCGGACATG

The closest primer to the end of the left border being LB4, LB1, LB2 and LB3 respectively. LB4 and LB1 primers sequence toward the left border. LB2 can sequence in either direction and LB3 sequences away from the left border.

### **7.3.5 Adaptor PCR**

A Clontech Genome Walker Kit (Biosciences, Palo Alto, USA) was used to isolate DNA flanking the activation tag. As explained in the introduction, the principle of the kit relies on two amplifications. Genomic DNA was digested using blunt end enzymes and ligated to an adaptor. PCR was carried out using the GeneAmp PCR system 2400 or 9600 (as outlined in the user manual) with primers designed from the plasmid pSKI015 and from the adaptor. The primary PCR mix was diluted and secondary PCR was done using nested primers. For the right border, RB1 was used for the initial PCR, then RB2 for the nested PCR. For the left border LB1 then LB2 was used. Fragments isolated were run on a 1.5% (w/v) agarose gel, excised and extracted using the Qiagen Gel extraction kit. DNA was ligated to the pCR2.1 vector and transformed into ultra competent cells as described below.

#### 7.3.5.1 Transformation

DNA isolated was run on a 1.5% (w/v) agarose gel. The band was cut out and DNA extracted using a Gel Extraction kit (Qiagen). DNA was checked again on an agarose gel. 6 µl of DNA was ligated into the pCR2.1 Vector (TA Cloning Kit, Invitrogen).

The ligation was catalysed by T4 DNA ligase (3 units) with a 1:1 ratio (sticky end reaction) or 3:1 ratio (blunt end reaction) of DNA to vector in the presence of 1X ligase buffer (10 mM MgCl<sub>2</sub>, 10 mM dithiothreitol (DTT), 0.5 mM ATP, 30 mM Tris:HCl, pH 7.8; Promega) and left for 1 ½ hours at room temperature and then overnight at 4 °C. The ligation mix was added to ultra competent cells according to the original TA cloning kit (Invitrogen).

Aliquots of transformed cells were smeared onto LB plates (1.0% tryptone, 0.5 % yeast extract, 1.0% NaCl, pH 7.0) (25 ml) containing ampicillin (40 mg/ml), GAL (80 ng/ml) and IPTG (0.4 mM). This was done near a bunsen burner on blue flame using sterile pipettes and an ethanol flame sterilised glass rod. Plates were left unsealed in a 37 °C cabinet overnight and examined in the morning for blue/white colonies. White (transformed) colonies were picked and cultured in liquid LB overnight at 37 °C on a shaker. Plates were sealed with parafilm and stored at 4°C. DNA was isolated from colonies grown in liquid LB using the QIAprep Spin Miniprep Kit. DNA was digested for 4 hours at 37 °C with *Eco*R1, which cuts out any DNA insert from the vector. The bands were run on a gel to check the size of the DNA insert. DNA was then sequenced.

### 7.3.5.2 Sequencing

Sequencing was carried out by the Wolfson Institute of Biomedical Research who used cycle sequencing which is based on the chain termination Sanger dideoxy method.

### 7.3.6 Inverse PCR

The principle of this technique is outlined in the Introduction. The DNA was digested and ligated as described below and a PCR was performed using the conditions outlined in the Clontech Genome Walker Kit manual. Bands were isolated from a 0.7% (w/v) agarose gel using a Gel Extraction Kit (Qiagen). DNA isolated was inserted into the pCR 2.1 vector for transformation using the TOPO TA cloning kit (Invitrogen).

#### 7.3.6.1 Digestion of DNA

The efficiency of the digestion was checked on a 0.7% (w/v) agarose gel: a smear should be seen. Enzymes were used with buffers supplied by the manufacturer. As a rule, 1 Unit of enzyme was used per  $\mu\text{g}$  of DNA (Table 12). For multiple digests the buffer that gave the best enzyme activity was used. Digests were run on a 0.7% (w/v) agarose gel and checked for a smear. After digestion was completed, reactions were stopped by incubating samples at 65°C for 15 min.

**Table 12.** Enzymes used for digestion and conditions used

Digestion enzyme	Buffer/Incubation temperature.
<i>Kpn</i> I	NEB Buffer 1/37 °C
<i>Hae</i> II	NEB Buffer 2/37 °C
<i>Hind</i> III	NEB Buffer 2/37 °C

### 7.3.6.2 Self-ligation of DNA

The amount of DNA was checked to be between 0.1 ng/μl to 20 ng/ml. 2 μl of the digestion mix was added to 1 μl of T4 ligase buffer (66 mM Tris-HCL pH 7.6, 1 mM DTT, 50 ng/μl BSA), 1 μl of T4 ligase (Boeringher) with 1 μl of ATP (10 mM) and 5.5 μl sterile water. Samples were left overnight at 16°C.

### 7.3.6.3 PCR conditions

PCR was done using the LB4 primer designed at the end of the left border sequence and the LB3 primer for *HaeII* and *KpnI*, but the LB2 primer for *HindIII*. This is as the *HindIII* enzyme would cut between the LB3 primer and the end of the left border. PCR was done using the Advantage Polymerase Mix (Clontech) and using the same PCR conditions out lined in the clontech Genome Walker Kit (Biosciences, Palo Alto, USA). Individual bands from the PCR were cut out, ligated into a vector and transformed into bacteria using the TOPO Cloning Kit (Invitrogen).

### 7.3.7 Plasmid Rescue

The principle of this technique is outlined in the Introduction (Figure 51). After digestion, fragments were ligated and transformed into bacteria via electroporation. Colonies were selected for ampicillin resistance. By using primers from the relevant border and the T3/T7 primers in bluescript, the DNA flanking the insert can be isolated. For the right border RB primers and the T3 primers were used for PCR, for the left border the LB primers and the T7 primers were used.

*PstI*, *HindIII* and *KpnI* were be used for right border plasmid rescue and *NotI*, *BamHI* and *SpeI* were used for left border plasmid rescue. *SacI* digests were used as a positive control and isolated the bluescript plasmid and some flanking pSKI015 sequence.

Digestion was carried out as before. Typically 20 µl of the digestion mix was used for the ligation, 10 µl of ligation buffer, 5 µl of ligase, 10 µl of ATP and 55 µl of sterile water. The ligation mix was diluted with water to minimise the likelihood of strands of DNA ligating to each other. The reaction was left overnight at 16°C and stopped in the morning by heating at 65°C for 15 min. The plasmids were transformed into cells by electroporation (heat shock with ultra competent cells was originally used but was ineffective as the competence of the cells was too low).

#### **7.3.7.1 Preparation of electrocompetent cells**

A plate of *E. coli* (DM125) was streaked and grown overnight. A single colony was taken and inoculated in 10 ml of SOB-Mg (Bacto-tryptone 20g/l, Bacto yeast extract 5g/l, NaCl 0.5g/l, KCl 0.19g/l, 5 M NaOH 0.2ml/l, to give pH7) in a 50 ml flask, which was left at 37°C in a shaker overnight. 5 ml of this was taken and inoculated in 500 ml of pre-warmed SOB-MG (in a 2l flask) and left at 37°C in a shaker until the OD 550 reached 0.75 (cell density is about  $3.6 \times 10^8$  cells/ml). Cells were decanted into a chilled 450 ml bottle and spun at 5000 rpm, 0°C for 10 min. The supernatant was removed and the pellet resuspended in 10 ml ice-cold 10% (w/v) glycerol. A further 390 ml of glycerol was added and mixed by inversion. The centrifugation and washing step was repeated. Finally, the pellet was resuspended in the last few drops of liquid and transferred to a disposable plastic tube where the volume was measured. A small aliquot was diluted one in 300 and the cell density was checked (final OD 550 = 200-250 units/ml). Aliquots of 100 µl were flash frozen in dry ice/ethanol bath and stored at -70°C.



### 7.3.7.2 Electro-transformation

1-2  $\mu$ l of DNA solution were added to 20  $\mu$ l of cells that were thawed on ice and then transferred immediately to a pre-cooled electroporation chamber (0.45 cm). Cells were electro-shocked at 2.5kV, 25  $\mu$ F, 200  $\Omega$ . Immediately, 1 ml of pre-warmed (to 37°C) SOC was added (Bacto-tryptone 20g/l, Bacto yeast extract 5g/l, NaCl 0.5g/l, KCl 0.19g/l, 5 M NaOH 0.2ml/l, to give pH7 make up to one litre, add 10 ml 2 M  $Mg^{2+}$  salt solution (1 M  $MgCl_2$  or  $MgSO_4$ ) autoclave and afterwards add 10 ml of 2 M sterile glucose and samples were kept at room temperature then plated on LB with ampicillin (100 mg/ml) to select for cells transformed with the insert. Plates were left to incubate overnight at 37°C.

### 7.3.8 Recovery of *Arabidopsis* DNA flanking insert

The size of *Arabidopsis* DNA isolated was determined by two methods.

1. Rapid colony PCR. This used the standard primers T3 and T7 which bind to the ends of bluescript. The reaction mix consisted of 2  $\mu$ l 10X Taq PCR buffer, 0.4  $\mu$ l dNTP (10 mM), 0.4  $\mu$ l of T3 primer, 0.4  $\mu$ l of T7 primer and 12.6  $\mu$ l sterile water. To this 0.4  $\mu$ l Taq Polymerase and 3.6  $\mu$ l of sterile water were added with the picked bacterial colony resuspended in it. DNA isolated was run on a gel with a ladder to determine the size of the insert. A high MW smear indicates that too many cells were picked, and there was too much template DNA. In this case, the experiment was repeated with fewer cells resuspended in the reaction mix.

2. Colonies were picked near a blue flame using a sterile needle. The needle was placed into a sterile tube containing 15 ml LB with ampicillin (100 mg/ml). This was left overnight in a shaker at 37°C. The QIAprep spin miniprep kit was used to isolate the plasmid DNA which was digested with the Sac I enzyme. This cut the insert into three fragments, one containing bluescript, one containing the BAR gene and one containing border DNA and the flanking DNA.

DNA was sequenced through the use of the T3/T7 primers and primers designed near the T-DNA border.

## 7.4 Results

To determine the flanking sequence three techniques were used: Adaptor PCR, Inverse PCR and Plasmid rescue. The mutant N31290D was used to test these methods to see which was the most effective.

To determine which enzymes were best to use in the digestion step in all three techniques, ten *Arabidopsis* BACs were examined. This allowed an estimate of the frequency of restriction sites of common enzymes (Table 13). The enzymes detailed below were used.

**Table 13.** Estimated frequency of restriction sites of common enzymes in *Arabidopsis*.

Enzymes	Res. Site	Total No. cuts	Average fragment length
<i>Bam</i> HI	G/GATCC	90	6966
<i>Dra</i> I	TTT/AAA	739	860
<i>Eco</i> RV	GAT/ATC	156	4019
<i>Hae</i> III	GG/CC	635	987
<i>Hind</i> III	A/AGCTT	321	1953
<i>Kpn</i> I	GGTAC/C	31	20225
<i>Pst</i> I	CTGCA/G	141	4447
<i>Pvu</i> II	CAG/CTG	141	4447
<i>Sma</i> I	CCC/GGG	19	32998
<i>Spe</i> I	A/CTAGT	116	5405
<i>Stu</i> I	AGG/CCT	44	14249
<i>Pme</i> I	GTTT/AAAC	43	14581
Total bp screened			626970

The frequency of restriction enzyme sites do not depend only on the number of base pairs in the restriction site. It is more related to the GC content. Perhaps restriction sites with a low GC content occur more as *Arabidopsis* is AT rich.

### **7.4.1 Adaptor PCR**

#### **Right Border**

N31290D mutant DNA was digested by four enzymes in the kit, *DraI*, *EcoRV*, *StuI* and *PvuII*. The PCR gave an 11 bp repeat which was homologous to the CaMV repeats at the right border. The end of the right border was estimated inaccurately and therefore the primers designed gave unsatisfactory results. As right border integration is often repetitive, it was easier to get flanking DNA from primers isolated from the left border.

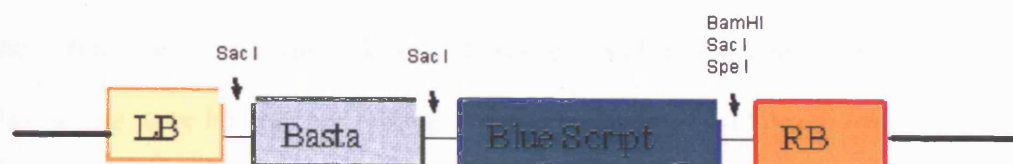
#### **Left Border**

N31290D DNA was digested by four enzymes in the kit, *DraI*, *EcoRV*, *StuI* and *PvuII*. *DraI* was the only enzyme to give a PCR band, unfortunately *DraI* cut too close to the left border to give any *Arabidopsis* DNA unique enough to align to a gene. However, the sequence obtained allowed the design of a new left border primer at the exact point of integration, LB4. Therefore, from the Adaptor PCR technique the end of the left border was determined, which allowed for a new primer to be designed. This primer is more useful as it is placed at the very end of the left border, therefore any sequence isolated will be from the sequence adjacent to the T-DNA insertion. This primer was used later, with inverse PCR.

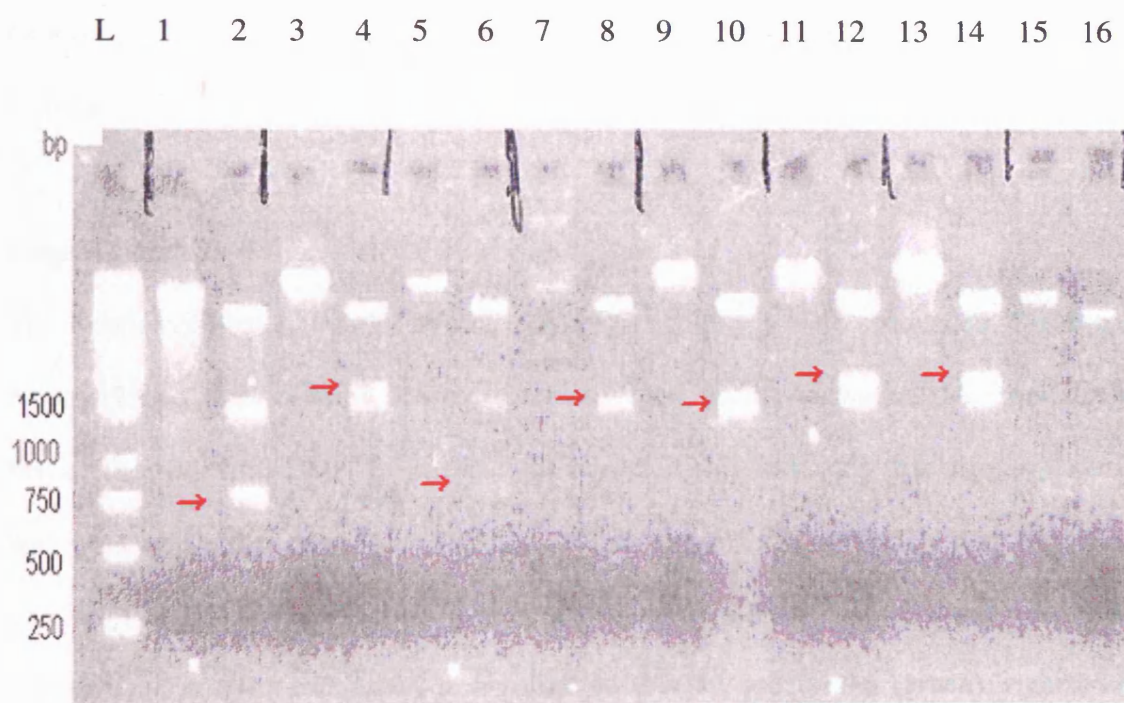
### **7.4.2 Left border plasmid rescue (LBPR)**

To try and obtain a larger fragment of *Arabidopsis* DNA adjacent to the T-DNA insert in the mutant N31290D, left border plasmid rescue was used. The *BamHI* and *SpeI* enzymes were used for LBPR. *SacI* was used as a positive control (to isolate bluescript and some flanking SKI015 DNA). Plasmids isolated were electroporated into bacteria. Plasmid DNA was isolated from each ampicillin-resistant colony and digested to see

whether plasmid rescue had been successful (Figure 52). Where in the T-DNA insert each enzyme is expected to cut is shown below. The length of flanking DNA can be determined by the length of DNA attached to the left border sequence.



**Figure 52** Where enzymes used for LBPR and the determination of isolated flanking region cut within the T-DNA insert.



**Figure 53.** Colony DNA from LBPR digests; (1) *Bam*HI colony 1, digested with *Bam*HI, (2) *Bam*HI colony 1, digested with *Sac*I, (3) *Bam*HI colony 2, digested with *Bam*HI, (4) *Bam*HI colony 2, digested with *Sac*I, (5) *Bam*HI colony 3, digested with *Bam*HI, (6) *Bam*HI colony 3, digested with *Sac*I, (7) *Bam*HI colony 4, digested with *Bam*HI, (8) *Bam*HI colony 4, digested with *Sac*I, (9) *Spe*I colony 1, digested with *Spe*I, (10) *Spe*I colony 1, digested with *Sac*I, (11) *Spe*I colony 2, digested with *Spe*I, (12) *Spe*I colony 2, digested with *Sac*I, (13) *Spe*I colony 3, digested with *Spe*I, (14) *Spe*I colony 3, digested with *Sac*I, (15) *Sac*I colony 1, digested with *Sac*I and *Kpn*I, (L) Ladder. Red arrows indicate bands including flanking DNA.

From this digest the following results were obtained:

**SacI colony DNA** – This was the control. The SacI digest produced a linear fragment. *KpnI* and *SacI* digest gave bluescript (2.8 bp) and a 725 bp fragment, which was expected.

**BamHI colony DNA** – All SacI digests showed the 3.5 kb fragment and 1.4 kb fragment that were from the T-DNA. Colony 1 and 3 also had an extra fragment. If 315 bp of that was left border, 700bp was flanking DNA. Colony 2 had an additional 1.6 kb fragment, which gave 1.3 kb of flanking DNA. Colony 4 may have had an addition fragment at 1.4 kb as this band is brighter, but it is hard to be sure.

**SpeI colony DNA** - All SacI digests showed the 3.5 kb and 1.4 kb fragments that were expected. Colony 1, 2 and 3 also gave a 1.6 kb fragment which gives flanking sequence of either 1.2 or 1.3 kb.

### Sequencing

The *BamHI* colony 1, *BamHI* colony 2 and *SpeI* colony 1 were sequenced. The *BamHI* colony 2 and the colonies derived from the *SpeI* digest showed DNA from the left border running into DNA from the right border. This indicated that a multiple insert was present in the mutant N31290D. When the *BamHI* colony 1 was sequenced, *Arabidopsis* DNA was located. Figure 54 shows DNA isolated from the mutant N31290D. *Arabidopsis* DNA is highlighted (black), left border (green), right border (red) and restriction enzyme sites (pink).

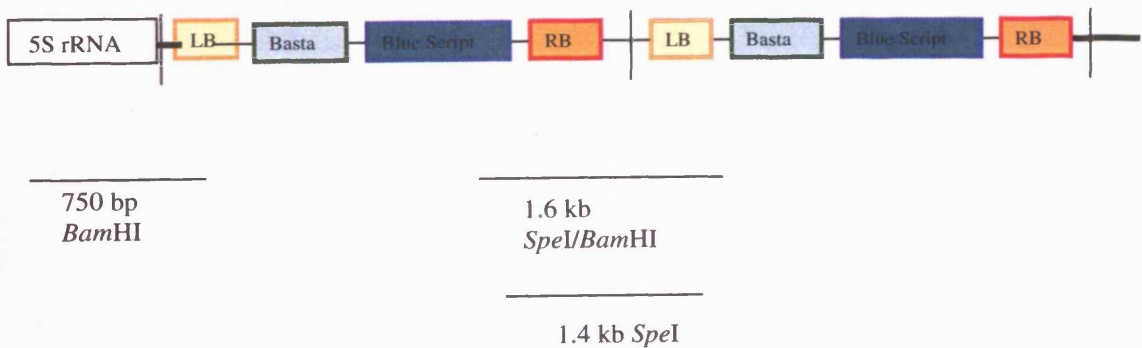
```
CGCGGTGGACGGCCGCTCTAGAACTAGTGGATCCGGTGCATTAGTGCTGGTATGATCGCATCCGTTAGT
ATATGCAATGCAATCGTATATATTCTTTTTTAAGTCTTGATGAACCGTTCGCCCTGGGTCCCACCCGCC
ATGTAGGGATACCCCATCTAGTCTTAACGAGCTTTGATGCATGAAAAATATTCGAAAACAATGCTTGAA
AAAGTAATTTTGGGTCCATCATATAGCACAAATCACGAAAATGCCCAAAAAAGTACTTAAAGGTCAAA
ATTGGAGTCGACAAAAAGTCAATGGAAAAGTTCCATTGTCTTGCTTCTTTCGTCGGAGGGCTCTCTTTG
```

GGCTTTCGAAAAGGTATCACAGGCCAAGTTTGGGCTCACGGTATAGAAGTTATTGAGTCATAAAGTTT  
TAACCAAAAAAAAAAAGGGATGCAACACGAGGACTTCATGTCCGGGAAATCTACATGGATCAGCAATG  
AGTATGATGGTCAATATGGAGGAAAAGAAAGAGTAATTACCAATTTTTTTTCAATTCAAAAATGTAGAT  
GTCCGCAGCGTTATTATAAAATGAAAGTACATTTGGATAAAACGACAAATTACGATCCGTCGTATTTAT  
AGGCGAAAGCCATAAAACAAATTATTCTATTTTCGGAAATCTTTATTTTCGACGTGTCTACATTCACGTCCA  
AATGGGGGGCTTAGATGAGAAACCTCACGATCGATATCTAGATCTCGAGCTCGAGATCTAGATATCGATA  
AGCCTGCATGCCTGCAGGGTCNGCCTGAGCCTCGACATGTTGTCCCAAAATCCGCCCTGAACCCGCC  
AACGAA

**Figure 54.** Sequence derived from colony 1 isolated by LBPR using *Bam*HI digest.

The sequence runs from the primer located at the right border end of bluescript into *Arabidopsis* sequence and then onto left border sequence. The *Arabidopsis* sequence aligns to 5S ribosomal DNA in the NCBI database. However, if the TAIR database is used the sequence matches to a gypsy-like retrotransposon (At4g06477), which has a 5S rRNA DNA insert at the end of its 4.3kb sequence.

There are approximately 1000 5S rRNA genes in *Arabidopsis* so it is unlikely that a change in this gene has caused a mutant phenotype. However, integration here could affect expression of a gene upstream or downstream from this fragment.



**Figure 55.** Alignment of all sequence data from LBPR.



Figure 55 summarises what was found from LBPR. Right border plasmid rescue may give a more unique flanking sequence to pin point exactly which 5S RNA has been disrupted.

#### **7.4.3 Right border plasmid rescue (RBPR)**

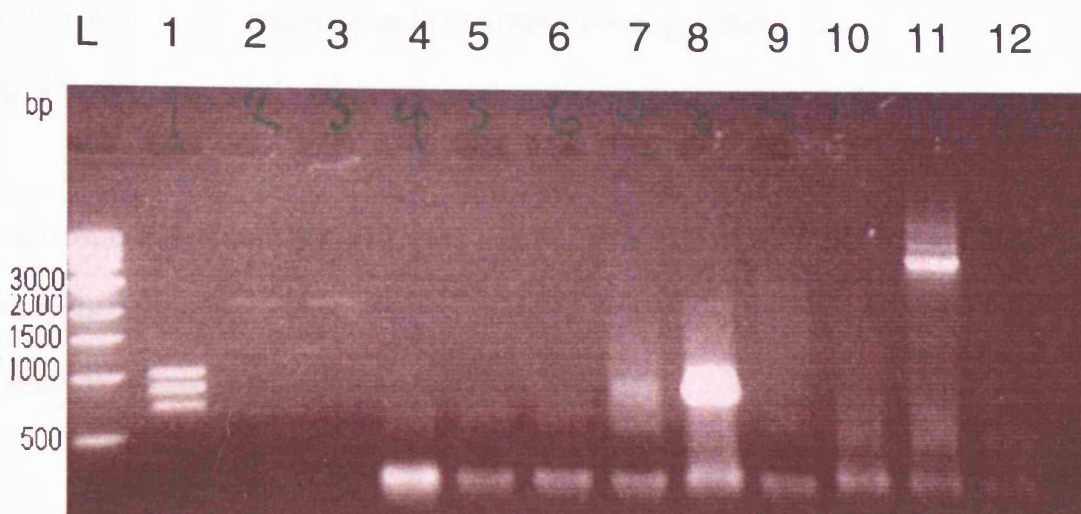
To try and determine more unique DNA from the N31290D, RBPR was used. This would hopefully yield DNA adjacent to the other side of the T-DNA insert. *KpnI* was used as it cuts once in the T-DNA, on the left side of bluescript. The sequence from the *KpnI* digest ran from the T-DNA into the right border CaMV repeat sequences. This confirms again that the N31290D mutant contains a multiple T-DNA insert.

#### **7.4.4 Inverse PCR**

Inverse PCR was used to obtain T-DNA insertion sites from the remaining mutants. Inverse PCR was also repeated with the N31290D mutant, this time using enzymes that would not cut between the T-DNA left border primers and the end of the 5S RNA sequence that was isolated.

Plant DNA from the mutants was digested with *HindIII*, *HaeII* or *KpnI* and inverse PCR carried out (Figure 56).





**Figure 56.** Fragments isolated through inverse PCR; Ladder (L), N31230G (1), N31290D (2), N31282A (3), N31253B (4), N31288C (5), N31230G (6), N31290D (7), N31288C (8), N31253B (9), N31230G (10), N31290D (11), N31230G (12).

This result confirmed that the T-DNA insert was present in all of these mutants (Figure 56). The fragments from lane 1, 2, 3, 7, 8 and 11 were cloned into the vector pCR2.1 from the TOPO TA cloning kit.

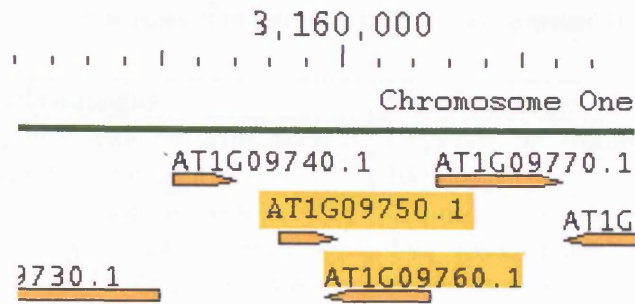
#### 7.4.4.1 N31290D

A 60 bp fragment was sequenced, of this 44 bp aligned to left border sequence and 16 bp aligned to *Arabidopsis* DNA. This is a shorter fragment than expected. The reason being that the PCR product was used directly in the transformation allowing for smaller fragments (which may not be visible on the gel in Figure 56) to be integrated into the vector. In future isolating DNA from the band to be cloned would yield better results. A BLAST on the whole NCBI database (including many different organisms) gave seven matches, all of which were in the *Arabidopsis* genome. There was a 1.3 kb identity in six of these entries, which may mean they were encoding the same protein. The seventh match (U2 snRNP-A') aligned to the others by 338 bp. The 16 bp sequence

was run in the TAIR database and DNA/RNA binding proteins came up with an E value of 0.009. These genes overlap and are found on chromosome one. The genes were:

1. Chloroplast nucleoid DNA-binding protein-related contains Pfam profile PF00026: Eukaryotic aspartyl protease; b similar to CND41, chloroplast nucleoid DNA binding protein [*Nicotiana tabacum*] At1g09750
2. U2 small nuclear ribonucleoprotein A, putative identical to U2 small nuclear ribonucleoprotein ' (U2 snRNP-A') [*Arabidopsis thaliana*] At1g09760

Although there was no significant alignment to anything else in the database, is it safe to say that this sequence is specific to these proteins in the *Arabidopsis* genome? When compared, these sequences overlapped in a 338 bp region. Therefore to test if these genes were causing the mutation, a primer could be designed in this region. If the consequent PCR results in an appropriately sized band (for instance if primers were designed to amplify a band of size X in the genomic sequence, should yield a band of size X+size of insert). To determine which of the matches was affected, primers could be designed for all 7 matches within the unique parts of their sequence.



**Figure 57.** The position of the two genes from the inverse PCR of N31290D on chromosome 1 (<http://www.arabidopsis.org/servlets/mapper?action=zoomto&band=0&zoomto=9>)

The exact position of Atg09750 is 3157503 - 3159147 bp and of Atg09760 is 3158748 - 3161666 bp. The T-DNA has inserted between these fragments and could affect either gene. If this position is confirmed with PCR primers designed in both these genes, then the enhancer tag could affect both of them (as the enhancer tag has a range of about 3.6 kb). RT-PCR or a Northern blot of genes in this region would allow us to see which genes are being enhanced.

#### 7.4.4.2 Other mutants

DNA isolated from inverse PCR showed that there was a multiple insert within the N31282A mutant. The T-DNA was integrated next to the bluescript fragment of a truncated T-DNA. For the other mutants, the DNA sequenced did not align to any unique *Arabidopsis* DNA.

### 7.5 Discussion

Protocols were perfected for adaptor PCR, inverse PCR and plasmid rescue. The advantages/disadvantages of each method are detailed in Table 14.

**Table 14.** Advantages/disadvantages of different techniques used to isolate DNA flanking the T-DNA insertion.

Method	Advantages	Disadvantages
Adaptor PCR	Quick, can be done with a variety of enzymes.	Depends on frequency of enzyme cut. Expensive
Inverse PCR	Quick, can be done with a variety of enzymes. Cheap, if Taq polymerase is used.	Cannot obtain high bp sequences with Taq polymerase, only with the more expensive Advantage Polymerase Mix (Clontech).
Plasmid rescue	Quick. Larger sequences can be obtained.	Requires electroporating equipment to get results. T-DNA must include a point of origin and selection gene. Enzymes can only be used that do not cut in such genes.

### 7.5.1 N31290D

It was shown that this mutant contained a T-DNA. Screening the F<sub>2</sub> generation showed that both the mutant phenotype and the BASTA resistance segregated with a 3:1 ratio. In this chapter, several methods were used to obtain the DNA sequence next to the T-DNA integration. It was shown that the N31290D mutant had a multiple insert that was integrated next to the sequence encoding 5S rRNA. Only 500bp of *Arabidopsis* DNA was isolated using plasmid rescue. As enhancers can affect up to 3.5 kb away from the insert, it would be interesting to obtain DNA which is further away from the inserts. This can be achieved by using a wider selection of enzymes.

As the sequence encoding 5S rRNA is repeated at least 1000 times in the *Arabidopsis* genome, it was difficult to obtain the exact chromosomal location of the insert. Cloix *et al.* (2002) showed that 5S rDNA sequence repeats are found in blocks in the genome. They commented that there are two types of 5S rDNA repeat, a complete one (about 500bp) and a truncated version and showed that there are single base pair differences in each repeated block.

The 5S rRNA sequence from N31290D aligned to a block in the centromere of chromosome 4. This block is 0.63 Mbp long and lies within 5.3 Mbp centromeric region of chromosome 4, at the upper moiety of a 2.7 Mb central region (containing transposable elements and repetitive sequences). It is unlikely that a different gene would be in this region whose expression could be affected by the insertion of enhancers. Therefore, the T-DNA insert could be affecting the expression of a gypsy-like transposon or alternatively a 5S rRNA. Is it possible that the enhancement of this 5S rDNA could cause the mutation? It has been shown that only two of the 5S rDNA array blocks of the *Arabidopsis thaliana* genome produce the mature 5S rRNAs, namely the chromosome 4 and chromosome 5 major blocks (Cloix, 2003). It also has been shown that polyamines bind to rRNA causing conformational changes (Amarantos *et al.* 2002). Furthermore, mutations that affect the synthesis of rRNA have shown a phenotype in plants. For example, the *Arabidopsis* mutation *rnrl*, which disrupts chloroplast rRNA synthesis, resulted in a decrease of chloroplast translational products, pale leaves, reduced growth rate and activities of photosystem I and II but high chlorophyll fluorescence (Kishine *et al.* 2004). Ammons *et al.* (2001) showed that deleting 5S rRNA genes results in greatly reduced growth rate in *E. coli*. Perhaps the enhanced root growth in N31290D was related to an increase in 5S rRNA.

Inverse PCR isolated a 16 bp fragment that aligned to seven *Arabidopsis* database entries, which had a 338 bp in common. Interestingly within these seven entries, sequences there were two overlapping genes, a DNA binding protein and a U2 small nuclear ribonucleoprotein A, which are both located on chromosome one.

It is thus likely that N31290D contained at least two mutations, one in chromosome four and one in chromosome one. However, it is unlikely that there is more than one whole

T-DNA insert in N31290D with different chromosomal locations, as BASTA resistance of the F<sub>2</sub> generation is 3:1. If there were more than one chromosomal location of the BAR gene, there would be more resistant plants. Therefore, it could either be that there are two linked T-DNA integration points or that there is an unlinked fragment of the T-DNA without the BAR gene inserted else where in the N31290D mutant genome. Tax *et al.* (2001) observed that chromosomal translocations are associated with the left border in *Arabidopsis*. They stated that even T-DNA insertion lines that exhibited Mendelian genetic behaviour could have a high frequency of T-DNA duplication and translocation. Either mapping or PCR methods can be used to detect rearrangements on T-DNA mutants.

#### **Further work with N31290D**

1. Design primers at the end of the 5S ribosomal gene. Design a primer in the gypsy-like retrotransposon (At4g06477) which is not homologous to the 5S ribosomal gene. To find out which of these is next to the T-DNA perform PCR using these primers and the LB2 primer in the left border of the T-DNA. LB2 is used as this primer is a palindrome and will work no matter which direction the T-DNA is inserted.
2. Design primers in both genes isolated from the inverse PCR experiment. Perform PCR using LB2 to confirm that a left border sequence is between these genes.
3. Design and use primers from the right border and primers designed in point one and two. The PCR product should be the same size as the number of bp in the T-DNA insert plus flanking DNA. This will determine if these genes are next to

a complete T-DNA insert or a fragment, and whether the enhancers are present in this chromosome location.

4. Use RT-PCR to look at the expression of all possible over expressed genes (within 3.6 kb of each confirmed T-DNA site) to see which gene is over/under expressed.
5. Backcrossing and selection on spermidine should result in one mutation within the genome.

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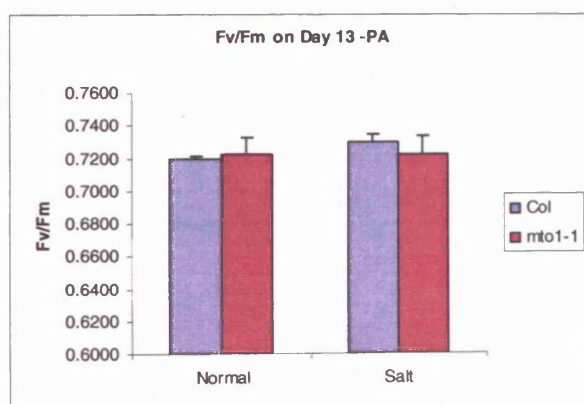
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# Appendix

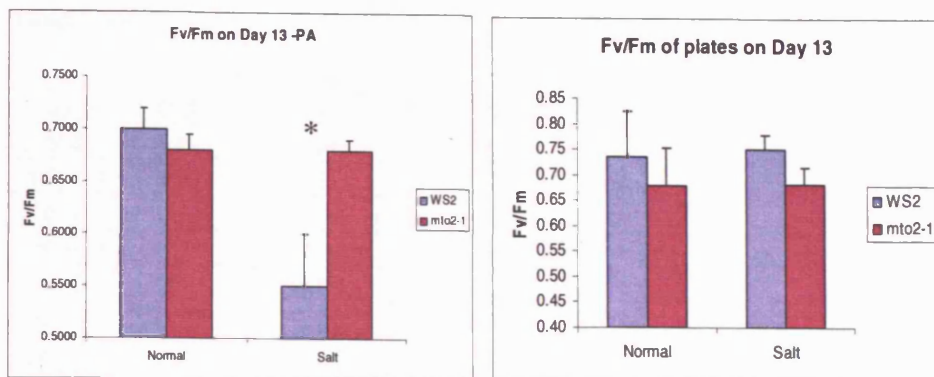
## Chapter three

**Table 15** Average fresh weight of *mtol-1* plants. SD = standard errors of 16 plants.

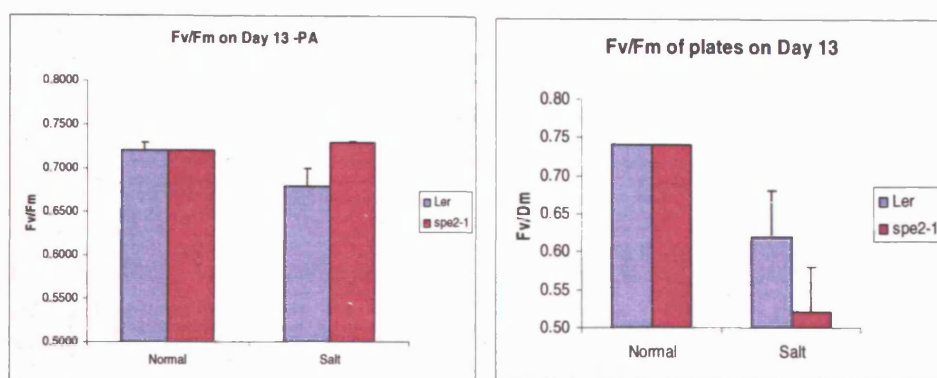
	Col-0	SD	mtol-1-1	SD
MS-MS	0.0440	0.0050	0.0120	0.0020
MS-125	0.0150	0.0020	0.0150	0.0110
100-125	0.0090	0.0020	0.0320	0.0190
	Col-0	SD	mtol-1-1	SD
MM	0.0900	0.0100	0.0200	0.0010
MS-125	0.0400	0.0100	0.0200	0.0010
100-125	0.0200	0.0100	0.0300	0.0010
	Col-0	SD	mtol-1-1	SD
MS-MS	0.0030	0.0004	0.0050	0.0015
100-125	0.0019	0.0004	0.0041	0.0011
	Col-0	SD	mtol-1-1	SD
MS-MS	0.0490	0.0050	0.0110	0.0020
MS-125	0.0150	0.0020	0.0150	0.0030
100-125	0.0070	0.0020	0.0320	0.0190
	Col-0	SD	mtol-1-1	SD
MS-MS	0.0041	0.0012	0.0030	0.0005
100-125	0.0068	0.0018	0.0135	0.0045



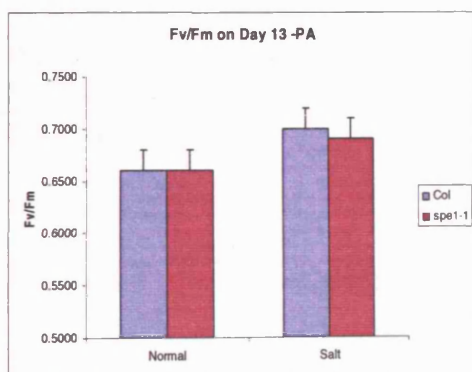
**Figure 58** Average  $F_v/F_m$  on Day 13. Error bars reflect standard error of 20 plants



**Figure 59** Average  $F_v/F_m$  on Day 13. Error bars reflect standard error of 20 plants. (\* = Difference between WS2 100 and mto2-1 100, P= 0.04).



**Figure 60** Average  $F_v/F_m$  on Day 13. Error bars reflect standard error of 20 plants



**Figure 61** Average  $F_v/F_m$  on Day 13. Error bars reflect standard error of 20 plants

**Table 16** (Spm+Spd)/Put ratio

	Normal	Salt
<i>mtol-1</i>	0.82	8.26
Col-0	4.52	4.40
<i>mtol-1</i>	3.20	0.29
Col-0	4.58	1.24
<i>mtol-1</i>	0.15	0.24
Col-0	0.16	0.08
<i>mtol-1</i>	4.39	9.28
Col-0	4.54	3.52

	Normal	Transfer	Salt
<i>mtol-2</i>	0.10	0.09	0.33
Ws-2	0.15	0.14	0.18
<i>mtol-2</i>	0.54	1.45	1.95
Ws-2	1.55	0.68	0.68
<i>mtol-2</i>	0.69	1.07	2.51
Ws-2	1.07	0.47	1.66

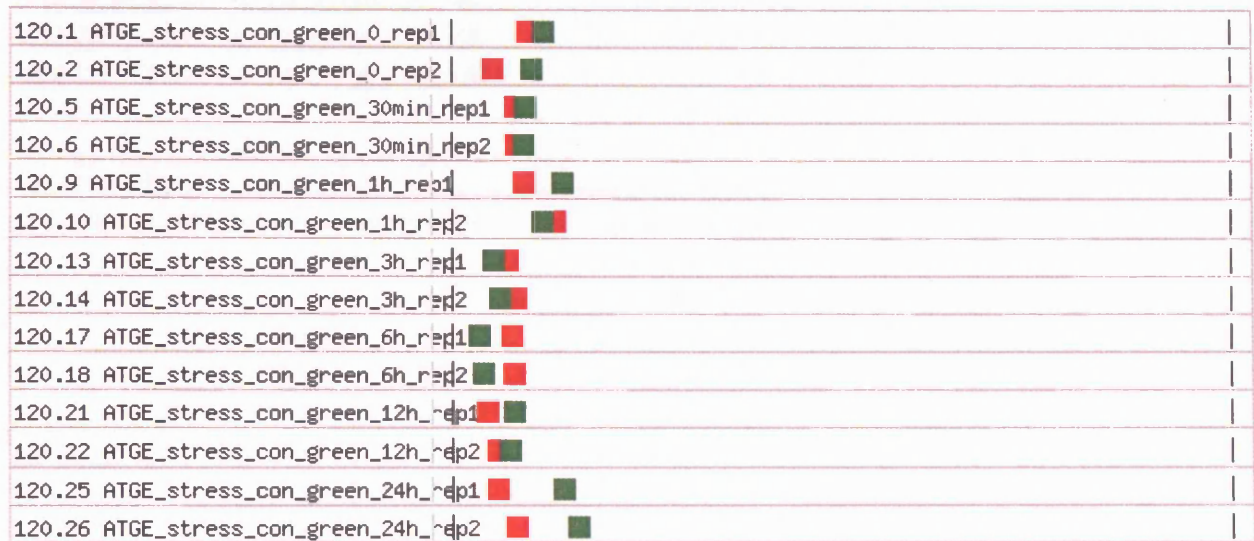
**Table 17** Correlation values

Polyamine – All data	Ler-0, <i>spe2-1</i>	Ws2, <i>mtol-1</i>	Col-0, <i>mtol-1</i>	Col-0, <i>spe1-1</i>
Spermine	R = - 0.29 P = 0.35	R = - 0.148 P = 0.65	R = - 0.38 P = 0.211	R = - 0.29 P = 0.17
Spermidine	R = - 0.208 P = 0.51	R = - 0.49 P = 0.05	R = - 0.78 P = 0.0001	R = - 0.41 P = 0.0508
Putrescine	R = - 0.44 P = 0.14	R = -0.043 P = 0.86	R = + 0.448 P = 0.06	R = + 0.61 P = 0.0017

Polyamine – Stressed	Ler-0, <i>spe2-1</i>	Ws2, <i>mtol-1</i>	Col-0, <i>mtol-1</i>	Col-0, <i>spe1-1</i>
Spermine	R = - 0.32 P = 0.67	R = + 0.129 P = 0.87	R = - 0.73 P = 0.26	R = - 0.19 P = 0.67
Spermidine	R = - 0.028 P = 0.97	R = - 0.66 P = 0.15	R = -0.78 P = 0.0001	R = - 0.47 P = 0.27
Putrescine	R = - 0.05 P = 0.86	R = + 0.53 P = 0.27	R = + 0.448 P = 0.0618	R = + 0.69 P = 0.08

Polyamine – Normal	Ler-0, <i>spe2-1</i>	Ws2, <i>mtol-1</i>	Col-0, <i>mtol-1</i>	Col-0, <i>spe1-1</i>
Spermine	R = + 0.28 P = 0.71	R = + 0.38 P = 0.61	R = - 0.26 P = 0.73	R = + 0.33 P = 0.4116
Spermidine	R = - 0.098 P = 0.9	R = - 0.76 P = 0.0042	R = + 0.57 P = 0.23	R = - 0.47 P = 0.23
Putrescine	R = - 0.93 P = 0.06	R = + 0.55 P = 0.24	R = + 0.662 P = 0.22	R = + 0.95 P = 0.003

## Chapter Four



























**Figure 62** Levels of *ADC1* (green) and *ADC2* (red) enzyme transcripts in plants grown under normal conditions. The figure was produced by analysing Affymetrix GeneChip data from the AtGenExpress experiments using Genevestigator (<https://www.genevestigator.ethz.ch/>).











122.17	ATGE_stress_osm_green_12h_rep1			
122.18	ATGE_stress_osm_green_12h_rep2			
122.21	ATGE_stress_osm_green_24h_rep1			
122.22	ATGE_stress_osm_green_24h_rep2			

**Figure 63** Levels of *ADC1* (green) and *ADC2* (red) enzyme transcripts in plants grown under osmotic stress conditions. The figure was produced by analysing Affymetrix GeneChip data from the AtGenExpress experiments using Genevestigator (<https://www.genevestigator.ethz.ch/>).

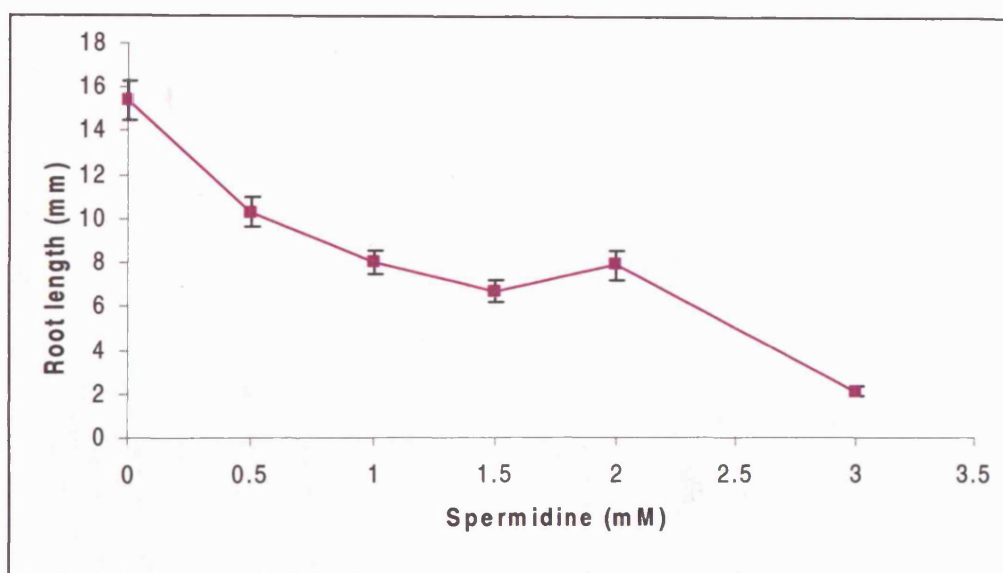
123.1	ATGE_stress_salt_green_30min_rep1			
123.2	ATGE_stress_salt_green_30min_rep2			
123.5	ATGE_stress_salt_green_1h_rep1			
123.6	ATGE_stress_salt_green_1h_rep2			
123.9	ATGE_stress_salt_green_3h_rep1			
123.10	ATGE_stress_salt_green_3h_rep2			
123.13	ATGE_stress_salt_green_6h_rep1			
123.14	ATGE_stress_salt_green_6h_rep2			
123.17	ATGE_stress_salt_green_12h_rep1			
123.18	ATGE_stress_salt_green_12h_rep2			
123.21	ATGE_stress_salt_green_24h_rep1			
123.22	ATGE_stress_salt_green_24h_rep2			

**Figure 64** Levels of *ADC1* (green) and *ADC2* (red) enzyme transcripts in plants grown under salt stress conditions. The figure was produced by analysing Affymetrix GeneChip data from the AtGenExpress experiments using Genevestigator (<https://www.genevestigator.ethz.ch/>).

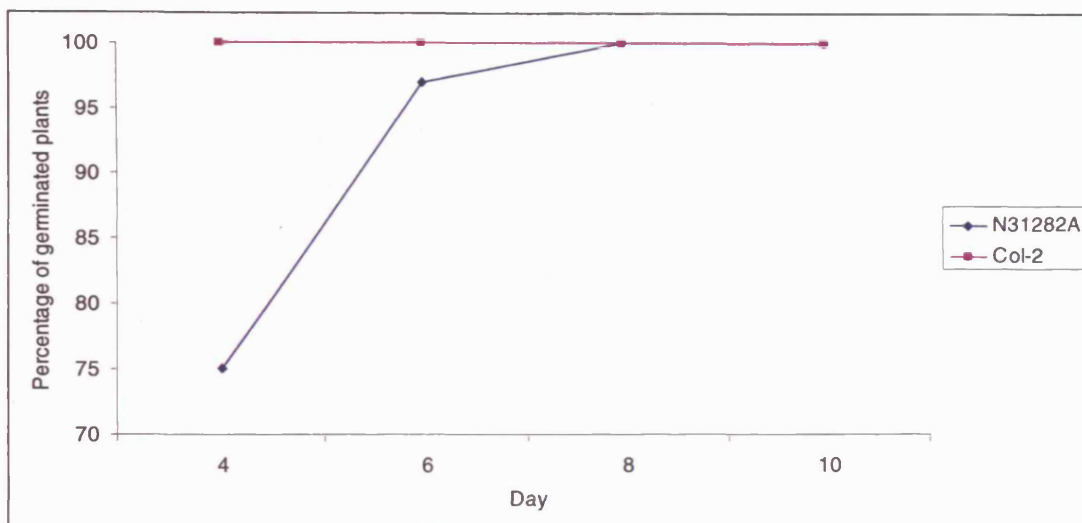
124.1	ATGE_stress_drought_green_30min_rep1			
124.2	ATGE_stress_drought_green_30min_rep2			
124.5	ATGE_stress_drought_green_1h_rep1			
124.6	ATGE_stress_drought_green_1h_rep2			
124.9	ATGE_stress_drought_green_3h_rep1			
124.10	ATGE_stress_drought_green_3h_rep2			
124.13	ATGE_stress_drought_green_5h_rep1			
124.14	ATGE_stress_drought_green_5h_rep2			
124.17	ATGE_stress_drought_green_12h_rep1			
124.18	ATGE_stress_drought_green_12h_rep2			
124.21	ATGE_stress_drought_green_24h_rep1			
124.22	ATGE_stress_drought_green_24h_rep2			

**Figure 65** Levels of *ADC1* (green) and *ADC2* (red) enzyme transcripts in plants grown under drought stress conditions. The figure was produced by analysing Affymetrix GeneChip data from the AtGenExpress experiments using Genevestigator (<https://www.genevestigator.ethz.ch/>).

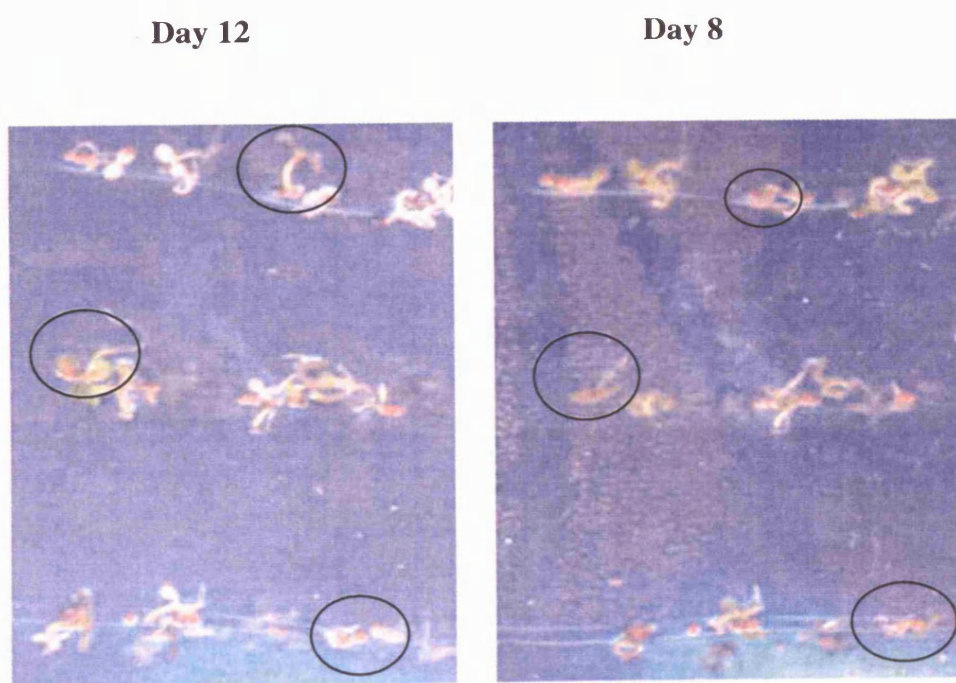
## Chapter five



**Figure 66** Root length of Col-2 on different concentrations of spermidine. Error bars reflect standard error of 10 plants.



**Figure 67** Delayed germination in N31282A mutants compared to Col-2 when grown on spermidine



**Figure 68** Early germination and bleaching are not linked in N31282A.